

Open Research Online

The Open University's repository of research publications
and other research outputs

Adoptive Transfer of HIV-Specific Cytotoxic T Lymphocytes

Thesis

How to cite:

Tan, Rusung (2000). Adoptive Transfer of HIV-Specific Cytotoxic T Lymphocytes. PhD thesis The Open University.

For guidance on citations see [FAQs](#).

© 2000 Rusung Tan

Version: Version of Record

Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.21954/ou.ro.0000f95d>

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

oro.open.ac.uk

Rusung Tan MD FRCPC

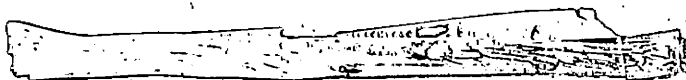
**Adoptive Transfer of HIV-Specific
Cytotoxic T Lymphocytes**

**A thesis submitted in fulfilment of the requirements for
the Open University for the degree of
Doctor of Philosophy**

Discipline: Immunology

February 1999

**The Institute of Molecular Medicine
John Radcliffe Hospital
Oxford**



DATE OF AWARD : 15 MAY 2000

ProQuest Number: C805116

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest C805116

Published by ProQuest LLC (2019). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Adoptive Transfer Of HIV-Specific Cytotoxic T Lymphocytes

Rusung Tan MD FRCPC

CONTENTS

| | |
|-----------------|----------|
| Abstract | 4 |
|-----------------|----------|

| | |
|-------------------------|----------|
| Acknowledgements | 5 |
|-------------------------|----------|

| | |
|----------------------|----------|
| Abbreviations | 7 |
|----------------------|----------|

Chapter One

| | |
|--|----------|
| The Cellular Immune Response To Viral Infection | 8 |
|--|----------|

| | |
|--|-----------|
| The Cellular Immune Response To HIV | 23 |
|--|-----------|

Chapter Two

**Adoptive Transfer Of Syngeneic Lymphocytes From An HIV-
Uninfected Twin To His HIV-Infected Sibling**

| | |
|---------------------|-----------|
| Introduction | 53 |
|---------------------|-----------|

| | |
|----------------|-----------|
| Methods | 56 |
|----------------|-----------|

| | |
|----------------|-----------|
| Results | 58 |
|----------------|-----------|

| | |
|-------------------|-----------|
| Discussion | 63 |
|-------------------|-----------|

Chapter Three

Adoptive Transfer Of Autologous HIV-Specific CTL To A Patient With A Low CD4 Count

| | |
|--------------|----|
| Introduction | 72 |
| Methods | 76 |
| Results | 86 |
| Discussion | 97 |

Chapter Four

Adoptive Transfer Of Autologous HIV-Specific CTL To A Patient With A Relatively High CD4 Count

| | |
|--------------|-----|
| Introduction | 103 |
| Methods | 104 |
| Results | 107 |
| Discussion | 111 |

| | |
|-------------------------------|------------|
| Summary of Experiments | 115 |
|-------------------------------|------------|

| | |
|-------------------|------------|
| References | 118 |
|-------------------|------------|

ABSTRACT

Several independent observations suggest that cytotoxic T lymphocytes (CTL) are critical for the control of HIV infection. We have studied the adoptive transfer of CTL in three patients with acquired immunodeficiency syndrome (AIDS).

In the first patient, we examined the CD8+ T cell repertoire before and after the transfer of syngeneic lymphocytes from his uninfected sibling to confirm whether aberrations exist in the CTL repertoire during advanced HIV infection and to determine whether adoptive immunotherapy with lymphocytes can lead to sustained expansions of CD8+ cells. Repertoire analysis revealed baseline expansions in some TCR subsets. Following cell transfer, there were new changes in two V-beta families, one at 24 hours post-infusion and the other and after 28 days. post-infusion. This study demonstrated that expansion and transient restoration of both CD4+ and CD8+ T-cells can occur *in vivo* following syngeneic cell transfer and that maximal lymphocyte expansion occurring appears to be maximal around 4 weeks post-infusion.

In the second and third patients, we studied the adoptive transfer of HIV-specific CTL clones. Despite substantial HIV-specific lytic activity *in vitro*, there were no significant changes in the virus load of patients following adoptive transfer. In one patient, we traced the fate of an infused clone using soluble MHC-peptide complexes and showed that cells were rapidly eliminated within hours of infusion, probably through apoptosis.

The use of CTL adoptive therapy in AIDS needs to be re-examined in light of these finding. Further trials of adoptive transfer of CTL should take into account the susceptibility of infused cells to *in vivo* apoptosis.

ACKNOWLEDGMENTS

First and foremost I would like to thank Patients AF, 008 and 868 who consented to these studies. Without their cooperation, this thesis and the figures inside it would not be possible. In particular, I would like to thank Patient 008, who is already the subject of several studies and numerous manuscripts. Aside from his prolific scientific output, he has the distinction of being the first human to undergo adoptive CTL transfer in the U.K.

Charles Bangham led me to England, found me a fabulous place to live and introduced me to Andrew McMichael. John Kurtz suggested that I meet Sarah Rowland-Jones. Andrew and Sarah became my supervisors and the best way I can think of to thank them is to say that it was worth completing this thesis just to get to know them better.

Experiments fail ninety-five percent of the time... or maybe it just seems that way. When your work consists of five percent success, you need some special qualities to persevere. One is a love of experimental science. Two is general optimism towards life. (This leads you to believe that the next experiment will work). Three, is workmates whose experiments also fail – I mean – whose unflagging support lifts you beyond the petty aggravations of negative data. Thus, I would like to thank Xiaoning Xu, Tao Dong, Gavin Screaton, Paul Bowness, Rachel Allen and Tom Hanke for making our bench space extremely crowded, interesting and fun.

I would also like to acknowledge the following individuals. Tao Dong and Veronique Braud taught me how to do a CTL assay. Steve McAdam provided me with the CTL clones from Patient 008. Philip Goulder suggested that Patient 868 might be a good candidate for adoptive transfer. James Wilson showed me how to spectratype TCR families and performed the flow cytometry for repertoire analysis. Pokrath Hansasuta prepared and froze lymphocytes from Patient 868. Tim Rostron produced the viral load results and Graham Ogg synthesized the A2-gag tetramer.

Thank you to Käthe Watanabe and Stan Riddell for sharing their cell culture tips with me and thank you to the clinicians involved with these cases – Drs. Chris Conlon, Tim Peto and Graz Luzzi and the late Dr. Rabindram.

My gratitude to you, Lorna (Carleton/Gardner) for your cheerful and uncomplaining secretarial assistance.

Finally, to Amy Cheng - thank you for disrupting your comfortable life in Vancouver. We're still married so it couldn't have been that bad.

ABBREVIATIONS

| | |
|-------------|---|
| AIDS | Acquired immunodeficiency syndrome |
| APC | Antigen presenting cell |
| ARMS | Amplification refractory mutation specific |
| AZT | Azidothymidine |
| β -2m | Beta 2 microglobulin |
| BCL | B cell line |
| BMT | Bone marrow transplantation |
| CAF | Cellular anti-viral factor |
| CDR | Complementarity determining region |
| CMV | Cytomegalovirus |
| COREC | Central Oxford Regional Ethics Committee |
| Cr | Chromium |
| CTL | Cytotoxic T lymphocyte(s) |
| DC | Dendritic Cell |
| DTH | Delayed type hypersensitivity |
| EBV | Epstein-Barr virus |
| ER | Endoplasmic reticulum |
| FDC | Follicular dendritic cell |
| GALT | Gut associated lymphoid tissue |
| Gran | Granzyme |
| HAART | Highly active anti-retroviral therapy |
| HBV | Hepatitis B virus |
| HC | Heavy chain |
| HCV | Hepatitis C virus |
| Hgb | Hemoglobin |
| HIV | Human immunodeficiency virus |
| HLA | Human leucocyte antigen |
| HTLV | Human T cell lymphotropic virus |
| LAK | Lymphokine activated killer cells |
| LCL | Lymphoblastoid cell line |
| LCMV | Lymphocytic choriomeningitis virus |
| MAC | Mycobacterium avium complex |
| MALT | Mucosa associated lymphoid tissue |
| MHC | Major histocompatibility complex |
| MMLV | Murine moloney |
| NSI | Non syncitium inducing |
| PBMC | Peripheral blood mononuclear cells |
| PCP | Pneumocystic carinii pneumonia |
| PCR | Polymerase chain reaction |
| Perf | Perforin |
| PHA | Phytohemagglutinin |
| PTLD | Post-transplantation lymphoproliferative disorder |
| R10 | RPMI with 10% fetal calf/human serum |
| RSV | Respiratory syncytial virus |
| SCID | Severe combined immunodeficiency |
| SI | Syncitium-inducing |
| SIV | Simian immunodeficiency virus |
| TAP | Transporter associated with antigen processing |
| TCR | T cell receptor |
| Th | Helper T cell |
| TIL | Tumour infiltrating lymphocytes |
| VSV | Vesicular stomatitis virus |
| VZV | Varicella zoster virus |
| WBC | White blood cells |

CHAPTER ONE

Introduction

The work presented in this thesis is an attempt to determine the safety, feasibility and efficacy of transferring autologous or syngeneic T-lymphocytes to patients infected with human immunodeficiency virus - 1 (HIV). By studying the effects of autologous or syngeneic lymphocyte transfer, we also hope to define better the role of cytotoxic T lymphocytes (CTL) in the pathogenesis of and the protection from acquired immunodeficiency syndrome (AIDS), to develop better protocols for the large scale *in vitro* production of T-cells and to elucidate the basic immunology underlying adoptive CTL therapy for infectious diseases or cancer. It is neither possible to understand the pathogenesis of AIDS nor the biology of CTL adoptive transfer without a general understanding of the human immune system. Thus, this introduction begins with an overview of basic immunology, with particular reference to MHC Class I restricted CTL responses to viral infections. This is followed by a more comprehensive review of the role of CTL in HIV infection.

The Cellular Immune Response to Viral Infection

The human immune system consists of adaptive and non-adaptive responses. The human immune system has evolved to protect healthy cells from potentially harmful micro-organisms such as bacteria, fungi, viruses and parasites. In addition, the immune system continually monitors and eliminates host cells which have undergone transformation and express tumour antigens.

Non-adaptive or innate immunity consists of phylogenetically primitive defense mechanisms which are common to both vertebrate and non-vertebrate organisms and which do not distinguish between a primary infection and subsequent infections (reviewed in (Medzhitov and Janeway 1997)). These mechanisms include external

barriers such as skin, sweat and hair as well as internal circulating cells such as polymorphonuclear cells, macrophages and natural killer cells, all of which provide a rapid and first-line response to infectious pathogens. In contrast, vertebrate organisms are able to generate a second line of defense termed adaptive immunity, which is both highly specific for the pathogenic organism and which displays long-term memory. The protein, carbohydrate and lipid components of foreign pathogens or tumours which provoke an immune response are known as antigens.

The immune system consists of specialized cells. All blood cells including those of the immune system derive from haematopoietic stem cells present in the bone marrow. These cells are termed pluripotent because they have the potential to differentiate into each diverse cell type which comprises blood. Pluripotent stem cells initially commit themselves to one of two cell lineages: myeloid stem cells which give rise to leucocytes (white blood cells), erythrocytes (red blood cells) and megakaryocytes (platelet precursor cells) or lymphoid stem cells which give rise to T and B lymphocytes (also known as T and B cells) as well as natural killer (NK) cells. Leucocytes comprise three polymorphonuclear cell types (neutrophils, eosinophils and basophils) as well as monocytes (mononuclear cells) and dendritic cells. Neutrophils engulf and destroy bacteria and fungi in a process termed phagocytosis; eosinophils assist in the killing of parasites while the natural function of basophils remains largely unknown. Monocytes in circulating blood are attracted to sites of inflammation within tissue (where they are termed macrophages or histiocytes) in order to capture antigens by phagocytosis and process them for delivery to T-lymphocytes. Similarly, bone-marrow derived dendritic cells (known simply as dendritic cells) act principally to process and display antigen and are critical for the activation of naive or unprimed T-cells.

B-lymphocytes are also able to process antigen for presentation to T-cells but in addition, they have the important capacity to differentiate further into plasma cells whose principal purpose is to secrete soluble protein receptors known as antibodies or immunoglobulin. Antibodies, which possess an almost limitless array of three-

dimensional receptor conformations, are capable of binding to soluble antigen thus targeting it for clearance by leucocytes or macrophages. In addition, some antibodies act by occupying antigen sites critical for host cell entry; these are known as neutralizing antibodies. The arm of the immune system mediated by antibodies is known as humoral immunity. In contrast, T-lymphocytes, utilize membrane-bound T cell receptors (TCR) to recognize antigen and rather than attaching to soluble antigen, TCR recognize and bind to small fragments of antigen that are bound to major histocompatibility complex (MHC) Class I or II molecules present on the surfaces of intracellularly infected cells or antigen presenting cells. T-lymphocytes may be divided into two broad groups: helper T-cells (Th) whose main function is to secrete soluble signalling proteins known as cytokines and cytotoxic T-cells (CTL) which act to kill infected or transformed cells. The defensive processes mediated by T-cells are termed cellular or cell-mediated immunity.

NK cells are a subset of cytotoxic lymphocytes which do not express a TCR. They were first proposed to recognize cells which do not express MHC Class I molecules on their cell surface by Karre et al (Karre, et al. 1986). The receptor which mediates the recognition and cytotoxicity by NK cells has not been identified. However, the inhibition of NK cell mediated target lysis has been shown to be mediated by the interaction of MHC Class I and Class I-like molecules with inhibitory receptors on NK cells (Colonna 1997, Colonna and Samaridis 1995, Wagtmann, et al. 1995, Braud et al, 1998).

Cells of the immune system originate, mature and interact in specialized organs.

Although all the cells of the immune system are derived from the bone marrow, interaction in other lymphoid organs is sometimes required for their optimal function. In particular, T-lymphocytes require passage through the thymus in order to mature fully and become capable of responding effectively to antigen. The bone marrow and the thymus, both sites of lymphocyte development and maturation, are known as central lymphoid organs. In contrast, lymph nodes are peripheral lymphoid organs which allow for the continuous physical interaction of T-cells, B-

cells and antigen during natural infection and are critical for the optimal generation of both humoral and cellular immune responses. Mature lymphocytes enter the cortex (outer layer) of lymph nodes via a number of afferent lymphatic vessels and exit the medulla (inner layer) via a single efferent vessel. The cortex consists primarily of resting B-cells interspersed with dense follicles known as germinal centres which contain proliferating B-cells and follicular dendritic cells (FDC). Follicular dendritic cells, not to be confused with bone-marrow derived dendritic cells, retain antibody-antigen complexes on their cell surfaces and are critical in positively selecting B-cells displaying high-affinity immunoglobulin receptors, a process termed affinity maturation. The paracortical region between the cortex and the medulla is an area where helper T-cells contact antigen presenting cells (APC) such as macrophages and dendritic cells while the medulla is populated primarily by macrophages and plasma cells.

Other peripheral lymphoid organs include the spleen which appears to function primarily to dispose of damaged or aged erythrocytes. Gross and microscopic examination of the spleen, reveals large areas of red cells known as the red pulp, within which are lighter areas of tissue known as white pulp. White pulp is composed of T-cells circumscribed by B-cells suggesting that at this anatomical site, B-cells also present antigen to T-cells and conversely, T-cells may signal B-cells. Other accessory lymphoid organs such as gut-associated lymphoid tissue (GALT), bronchial-associated lymphoid tissue (BALT), mucosal-associated lymphoid tissue (MALT), tonsils, adenoids, appendix and Peyer's patches have slightly less organized histological anatomy than lymph nodes but subserve similar functions. All peripheral lymphoid organs serve as meeting places for lymphocytes and antigen presenting cells.

T-lymphocytes are defined by the expression of a clonal surface T-cell receptor. As discussed above, all T cells are defined by their expression of a clonal TCR. The TCR is a transmembrane glycoprotein composed of either α and β (Hedrick, et al. 1984, Yanagi, et al. 1984) or γ and δ (reviewed in Chien, et al. 1996) heterodimeric

chains. (This thesis deals entirely with T lymphocytes bearing $\alpha\beta$ TCRs.) The extracellular domain of each chain contains a highly polymorphic and exposed face comprising the complementary determining regions 1, 2 and 3 (CDR 1,2 and 3) which allow for the recognition of a vast number of antigenic structures. The nucleotide and amino acid sequences of CDR 1 and 2 are less polymorphic than CDR3 and the crystal structure of MHC-peptide-TCR complex confirms that they bind to the less polymorphic regions of the MHC molecule, while the highly polymorphic CD3 region binds to the centre of the peptide-binding site of Class I molecules (Garboczi, et al. 1996, Garcia, et al. 1996). The cytoplasmic domain of the TCR associates with a multimeric signalling complex, CD3, itself consisting of several protein chains of which the ζ -chain is particularly important. The ζ -chain contains tyrosine-phosphorylation sites which are critical in transducing the TCR signal. Upon binding of the TCR by MHC-peptide, the phosphorylated ζ -chains are able to bind and activate a 70 kD cytosolic tyrosine kinase called zeta-associated protein (ZAP-70) (Chan, et al. 1992). and initiate a downstream signalling cascade resulting ultimately in activating the effector functions of the lymphocyte.

Two additional cell surface glycoproteins, the CD4 and CD8 differentiation markers, define the two (helper and killer) subsets of T-lymphocytes. As mentioned, CD4+CD8- T-cells (helper T-cells) recognize antigen which is bound to MHC class II molecules on the surface of professional APC. Activation of helper cells results in the secretion of cytokines which bind to their counter-receptors on the surface of neighbouring cells, stimulating them to carry out effector functions. Thus cytokines act as local area hormones. Depending on the type of cytokines secreted, CD4+ cells may be termed Type 1 (Th1), Type 2 (Th2) or Type 0 (Th0). Th1 cells predominantly secrete IL-2 and gamma-interferon in response to IL-12 and preferentially stimulate a cellular response to infection while Th2 cells primarily secrete IL-4,5,10 preferentially tilting the immune response towards antibody production (reviewed in (Mosmann and Sad 1996)). Th0 cells secrete neither or a mixture of both types of cytokines. It should be noted that in humans, there is not as clear a distinction between Th1 and Th2 responses as in mice.

Maturation of T-lymphocytes occurs in the thymus. Immature T-lymphocytes from the bone-marrow migrate to the thymus (in which they are known as thymocytes) where they evolve to acquire a mature CD3+CD4+ or CD3+CD8+ phenotype. Generation of the TCR is a complex process involving the germline recombination of different gene segments. Each α or β chain of the TCR is encoded by gene segments which consist of a V (variable), a J (joining) and a C (constant) region. In addition, β chains have a D (diversity) segment. The TCR β -chain genes rearrange first in CD4-CD8- immature thymocytes (double negative thymocytes). Expression of Rag-1 and Rag-2, genes which mediate germline rearrangement, produces first a D-J rearrangement, followed by a VDJ and finally a VDJC encoded chain. Expression of the β -chain on the immature lymphocyte surface in complex with a surrogate, pre- α chain and a CD3 complex signals the thymocyte to express CD4 and CD8 genes and begin α -chain rearrangement. The α -chain rearrangement proceeds until an α -chain capable of efficient pairing with a β -chain is produced. At this stage the cells exhibit a CD3+CD4+CD8+ phenotype and are known as double positive thymocytes and are ready for negative and positive selection. Negative selection refers to the selective death of potentially autoreactive T-cells whose TCR recognize, with sufficient affinity, self-antigen in complex with self MHC molecule. This process is mediated by bone marrow derived dendritic cells which express MHC Class I and Class II molecules in complex with self-peptide. When the dendritic cells encounter double-positive thymocytes in the thymus, those T-cells whose TCR binds to MHC self-antigen with a high affinity are triggered to undergo programmed cell death via a physiological mechanism termed apoptosis. T-cells which survive negative-selection may then be positively selected by exposure to self MHC molecules expressed on cortical thymic epithelial cells. If the TCR is able to recognize MHC-self peptide with low affinity and the selecting molecule is Class II then CD3+CD4+ cells are selected; if the selecting molecule is Class I, CD3+CD8+ cells are positively selected. These mature, single-positive thymocytes are then ready to leave the thymus and enter the peripheral circulation as naive lymphocytes.

Double-positive cells whose TCR cannot bind any self MHC molecules are neither positively nor negatively selected and die by apoptosis in the thymus. The entire family of mature T-cells which leaves the thymus is known as the T-cell repertoire.

Lymphocyte recognition of antigen. The adaptive immune response begins when lymphocytes expressing clonal T-cell receptors (TCR) bind to either MHC class I or class II molecules bearing antigenic peptide. CD4⁺ lymphocytes bind only to Class II complexes while CD8⁺ lymphocytes bind only to Class I complex. In addition, lymphocytes generally recognize only self-MHC in a phenomenon now termed MHC restriction. (Zinkernagel and Doherty 1974, Zinkernagel and Doherty 1975, McMichael, et al. 1977). In the case of CTL, TCR recognition of Class I complex initiates a series of intracellular events beginning with recruitment of Zap-70 and ending with the destruction of the peptide-bearing target cell. The initial demonstration of MHC Class restriction was followed by experiments showing that CTL recognize fragments of antigenic protein in complex with Class I molecules on the surface of virus infected cells (Townsend and Bodmer 1989, Townsend, et al. 1986). These data were ultimately confirmed by X ray crystallographic studies of several human and murine class I molecules (Bjorkman, et al. 1987).

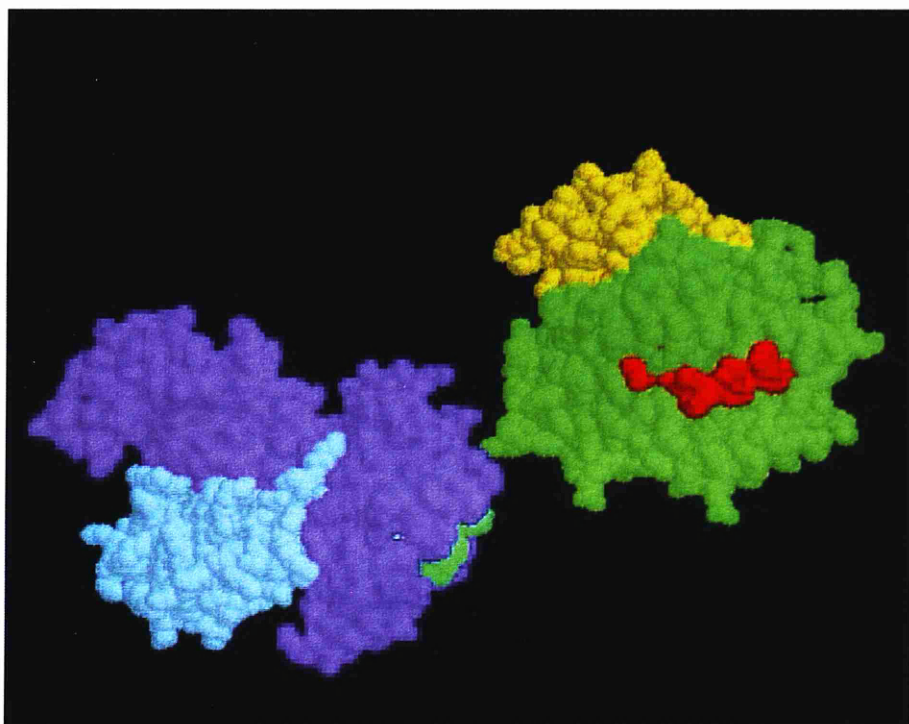
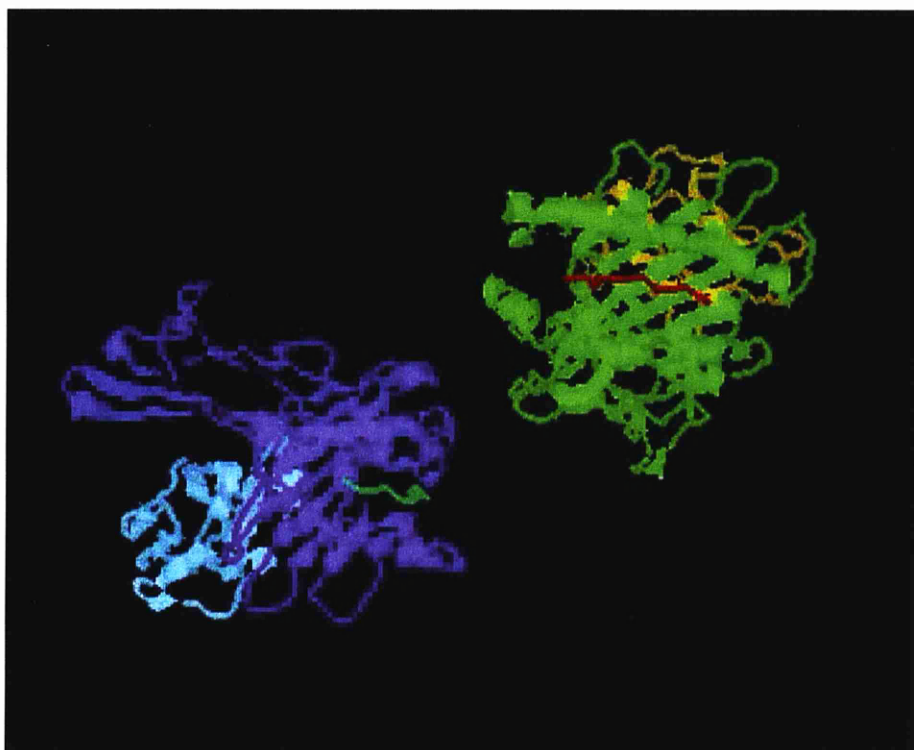
MHC Class I molecules consist of a 45kD polymorphic transmembrane heavy chain and a smaller 12kD non-polymorphic chain, beta-2 microglobulin (β -2M). The heavy chain comprises three domains, α 1, α 2 and α 3. The α 1 and α 2 domains form a beta-sheet platform upon which rests diagonally two highly polymorphic alpha helices, together forming the floor and sides of the peptide-binding groove (Figure 1). The ends of this groove in Class I molecules are closed and provide hydrogen-bonding sites for the N and C-terminal domains of the peptide thus burying the peptide at both ends and limiting the size of most Class I peptides to 8-10 amino acids (Madden, et al, 1993; Saper, et al, 1989). Longer peptides binding in the groove may do so by bulging the central portion of the peptide outwards or in exceptional cases the N or C-terminal domains may extend from the outside pockets as they do in MHC Class II complexes. Numerous hydrogen bonds may be formed

between the peptide and the heavy chain along the length of the groove, accounting for the broad diversity of peptides capable of binding to given alleles of class I molecules. Specific peptide binding sites or pockets vary from allele to allele, preferentially binding particular amino acids which are thus termed anchor residues. Similarly, certain amino acids of the peptide point their side chains out of the groove where they are free to interact with the T-cell receptor.

Figure 1: Crystal structure of HLA-A2

The structure of the MHC Class I molecule, HLA-A201 complexed with a peptide epitope from HIV Pol (ILKEPVHGV) displayed in ribbon (top) and spacefill (bottom) formats viewed from two directions. The peptide binding groove (green), formed by the $\alpha 1$ and $\alpha 2$ helical domains of the heavy chain holds the nonameric peptide (red).

Figure 1: Structure of HLA A*0201



Antigen-presenting cells process and display antigen for helper T-cells.

All nucleated cells normally express surface MHC Class I molecules and thus may be recognized by CTL when infected by intracellular pathogens. Class II MHC expression however is limited to a subset of specialized cells termed antigen-presenting cells (APC). APC include macrophages, B lymphocytes and dendritic cells and generally, only these cells may be recognized by CD4+ helper T cells. In addition to MHC Class II molecules, APC often express co-receptors, which upon binding their counter-receptor on CD4 cells, provide a "second signal" which appears to be necessary for optimal CD4 T-cell activation. The requirement for a second signal presumably adds an additional layer of safety to the immune response and thus may play a critical role in preventing autoimmune responses. Self antigens which are recognized by lymphocytes expressing aberrant TCR's (which have escaped negative selection) may still be rendered anergic (that is, made incapable of responding to antigen) if they are activated in the absence of a proper second signal. A number of accessory or adhesion molecules expressed on APC's may deliver a second signal and also increase the avidity and affinity of the lymphocyte-APC interaction but the most important of these appear to be CD80/86 (B7) which binds to CD28 expressed on T-cells. However, mice deficient in CD28 are still capable of mounting seemingly normal lymphocyte responses suggesting that alternative pathways exist (Shahinian, et al. 1993). Bone-marrow derived dendritic cells (DC) appear to be particularly potent APC probably because of their high expression of both MHC Class II and CD80 molecules (Tan, et al. 1992) and are the principal activators of naive T cells.

Antigenic intracellular proteins are degraded into small fragments in the cytosol. As mentioned previously, protein antigens are not presented in whole by MHC molecules but rather are degraded into short peptides before association with class I or class II molecules (Townsend and Bodmer 1989); (Germain 1991). This process begins in the cytosol where translated viral and bacterial proteins as well as tumour derived proteins are tagged with ubiquitin and targeted for degradation by a proteasome complex consisting of two subunits, LMP2 and LMP7 (Goldberg and

Rock 1992). Proteasomal products, typically oligopeptides 3-15 amino acids in length are actively translocated from the cytosol into the endoplasmic reticulum (ER) by a membrane bound transporter-associated with antigen processing protein (TAP), itself composed of two subunits, TAP1 and TAP2 (Trowsdale, et al. 1990). Peptide fragments associate with newly synthesized class I heavy chains in the ER and are stabilized by association with beta-2 microglobulin before trafficking to the Golgi apparatus and then to the cell surface. In comparison, MHC Class II-associated peptides are normally derived from extracellular proteins taken into professional APC by phagocytosis or endocytosis and digested in lysosomes by enzymes known as cathepsins. These peptide fragments, generally longer than those on Class I molecules, are bound to MHC Class II molecules in special endosomal compartments before export of the complexes to the cell surface (reviewed in Germain 1991). This apparent compartmentalization of MHC Class I and II antigen processing is not absolute and endocytosed extracellular antigen can sometimes be shunted to the Class I processing pathway (Rock, 1996).

Effector functions of CTL.

When the TCR which are present on CTL recognize and engage MHC Class I-peptide complexes, a series of intracellular signalling events are initiated culminating in the destruction of the antigen-bearing cell. This occurs through two distinct mechanisms (Figure 2). The main pathway of cytotoxicity is explained by the granule exocytosis model (Henkart 1994, Podack, et al. 1991) and involves the secretion of soluble, pore-forming proteins, perforin as well as the serine proteases, such as granzymes. Expression of perforin is generally confined to CTL, NK cells and $\gamma\delta$ T-cells. Upon CTL activation, preformed cytoplasmic granules containing perforin subunits are released into the extracellular compartment where Ca^{++} dependent conformational changes allow perforin monomers to integrate and polymerize in the target cell membrane. These pores allow for the entry of granzymes which initiate an apoptosis signalling cascade. Perforin cytotoxicity, however, cannot be the sole mechanism of CTL directed lysis since perforin-deficient mice are also able to lyse lymphoid cells (albeit with a reduced efficiency)

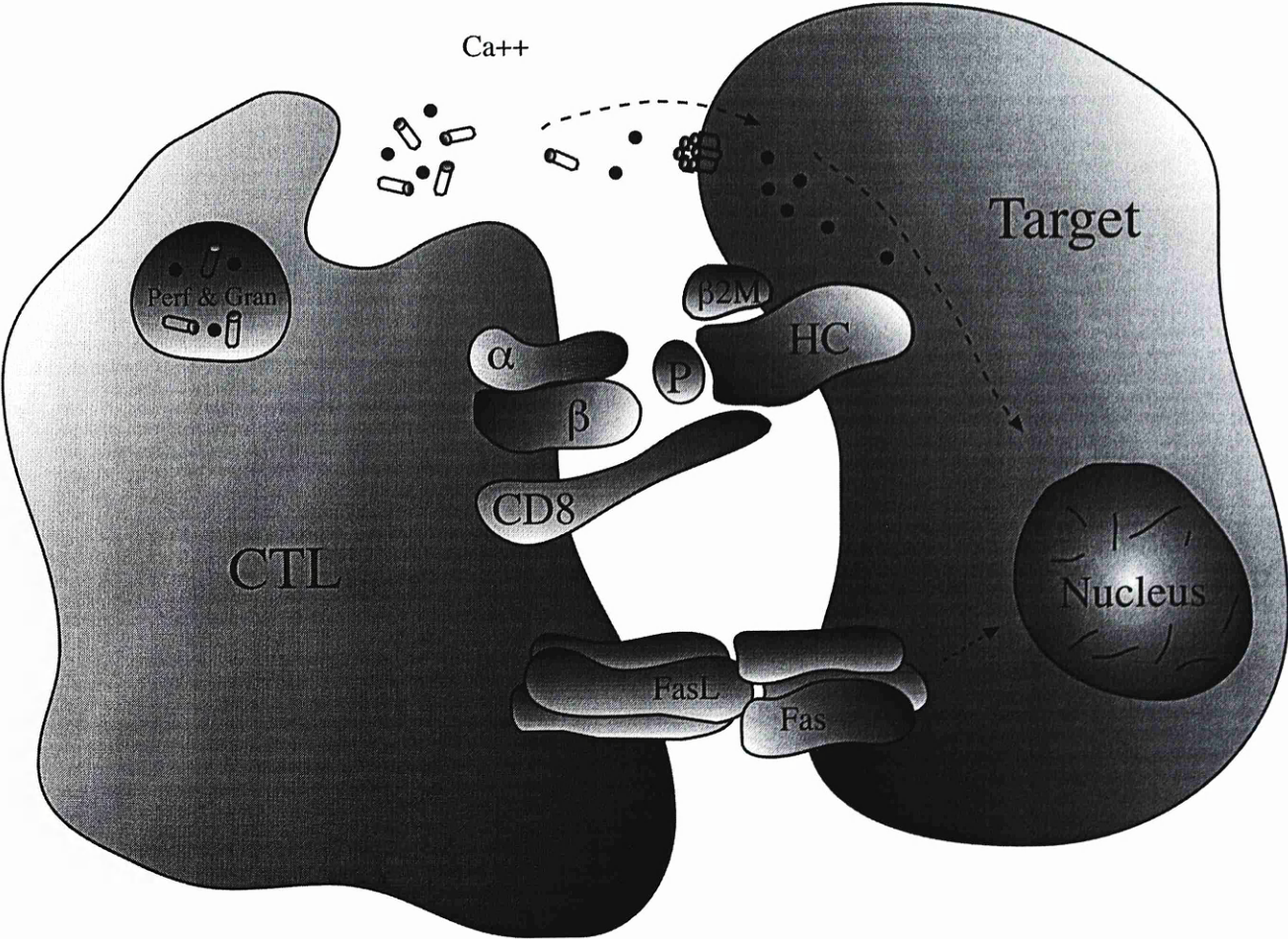
(Kagi, et al. 1995, Rouvier, et al. 1993, Walsh, et al. 1994). The second pathway of CTL induced death was found to involve the interaction of Fas on target cells with its ligand FasL on CTL.

Fas (CD95), a member of the tumour necrosis factor receptor family, surface molecule containing a cytoplasmic death domain. Binding of Fas either by antibody or by its natural ligand, FasL results in the initiation of apoptosis. Cells such as primary fibroblasts do not express Fas and hence when used as target cells rely entirely on the perforin pathway for cytotoxicity. Lymphoid cells however, express Fas accounting for their susceptibility in perforin-deficient mice. When perforin-deficient mice were crossed with *lpr* mice, which bear a natural Fas mutation, no CTL activity could be detected even on lymphoid cells demonstrating that these two pathways account for all of the cytotoxicity of CD8⁺ cells. Both perforin-dependent and Fas-dependent death are the result of apoptosis of the target cell. However, caspase-inhibitors which block the signalling cascade necessary for apoptosis of the target cell do not prevent its death by lysis (Sarin, et al. 1997). Thus, perforin alone may lyse target cells but granzymes and Fas are necessary for apoptosis. Aside from directly killing target cells, activated CTL also release tumour necrosis factor alpha (TNF α), which potentiates killing, and gamma interferon (IFN- γ) which renders neighbouring cells more resistant to viral infection. The CTL which initially expand on antigen contact can persist as memory cells. Recently, Wagner et al have reported that two anti-viral chemokines, macrophage inflammatory protein (MIP)-1 α and RANTES may also be released with granzymes by CTL (Wagner et al).

Figure 2: CTL recognition and effector function.

The clonal $\alpha\beta$ T-cell receptor (TCR) on a cytotoxic T lymphocyte (CTL) recognizes antigenic peptide (P) in association with an MHC Class I molecule (HC= heavy chain, $\beta 2m$ - beta 2 microglobulin). Upon recognition, the CTL is triggered to release perforin (Perf) and granzymes (Gran). In the presence of Ca^{++} , perforin polymerizes in the target cell to form pores which allow entry of proteolytic granzymes which trigger a signal cascade (dotted arrow) resulting in apoptosis of the target cell. Activated CTL also express Fas ligand (FasL) and can trigger Fas-dependent apoptosis in cells expressing Fas.

Figure 2: CTL recognize and kill target cells via two pathways



CTL act to limit virus replication.

MHC class I restricted CTL were first shown to play a critical role in regulating virus infections in mice infected with lymphocytic choriomeningitis virus (Zinkernagel and Doherty 1974, Zinkernagel and Doherty 1975) although in this infection they act to mediate the immunopathology of the disease (Buchmeier, et al. 1980). CTL were subsequently detected in murine influenza virus infection (Zweerink, et al. 1977), and it was demonstrated that the adoptive transfer of CTL clones produced potent anti-viral effects (Braciale, et al. 1981, Lin and Askonas 1981, Lukacher, et al. 1984). Similar observations were made for respiratory syncytial virus (RSV) (Cannon, et al. 1988) and herpes simplex infection in mice (Bonneau and Jennings 1990).

CTL have been detected in many human virus infections (Bangham and McMichael 1989), although evidence for a protective role in these infections has been harder to obtain. In influenza, CTL levels correlated with protection from deliberate infection of volunteers (McMichael, et al. 1983) and in immunosuppressed patients following bone marrow transplantation (BMT), CTL levels correlate with protection from cytomegalovirus (CMV) infection (Reusser, et al. 1991). In addition, the transfer of CMV-specific CTL appears to protect BMT patients from CMV disease (Walter, et al. 1995).

Adoptive transfer of T-cells has also been shown to induce regression of post-transplantation lymphoproliferative disorders (PTLD), almost all of which are associated with Epstein-Barr virus (EBV). Following acute EBV infection, the virus persists latently in some B cells switching its gene expression pattern such that only the nuclear antigen, EBNA-1 (Rowe, et al. 1987, Young, et al. 1989) is translated. Thus the latent virus escapes CTL detection because EBNA-1 is normally not processed in the MHC Class I pathway. Changes in the viral gene expression program involving the breakthrough synthesis of non-EBNA-1 proteins and transformation of lymphoid cells is controlled by a strong lifelong memory CTL response. However loss of CTL function by immunosuppression allows for the

development of PTLTD (Beral, et al. 1991, Rowe, et al. 1991). The role of CTL in preventing uncontrolled EBV-driven lymphoproliferation has been strongly supported by recent reports that it is possible to treat some EBV-related lymphomas by the transfer of donor derived leucocytes or EBV-specific CTL (Papadopoulos, et al. 1994, Rooney, et al. 1995).

Persistent viruses have strategies to escape recognition by CTL. The importance of CTL in the control of viral infections is also underscored by the findings that nearly all persistent viruses studied to date, including most of the human herpesvirus family, possess mechanisms for escaping CTL detection and destruction. For example, Herpes simplex virus produces a protein ICP-47 which binds TAP preventing translocation of peptides to the ER (Fruh, et al. 1995, Hill, et al. 1995, York, et al. 1994). Human cytomegalovirus (CMV) expresses at least five protein products which help the virus evade CTL. US2 and US11 each shunt newly synthesized Class I molecules from the ER to the cytosol where they are degraded (Wiertz, et al. 1996, Wiertz, et al. 1996). Despite homology to US2 another CMV early gene product US3 does not degrade Class I complexes but retains them in the ER (Ahn, et al. 1996, Jones, et al. 1996). Two other gene products UL18 and m144 are class I homologs and can engage NK inhibitory receptors preventing NK cell lysis. Most recently, Gilbert et al have shown that the CMV viral matrix protein pp65 has Ser/Thr kinases activity and phosphorylation of the principal immediate early protein, p72 prevents its recognition by CTL (Gilbert, et al. 1996). Finally, as mentioned, EBNA1 of EBV also avoids antigen presentation, probably by blocking its proteasomal degradation (Levitskaya, et al. 1995). There is also some evidence that HIV accessory proteins may enable evasion of the immune response as both Vpu and Nef appear to downregulate expression of MHC Class I molecules in HIV infected cells (Kerkau, et al. 1989, Collins, et al. 1998).

The Cellular Immune Response to HIV

The biology of HIV

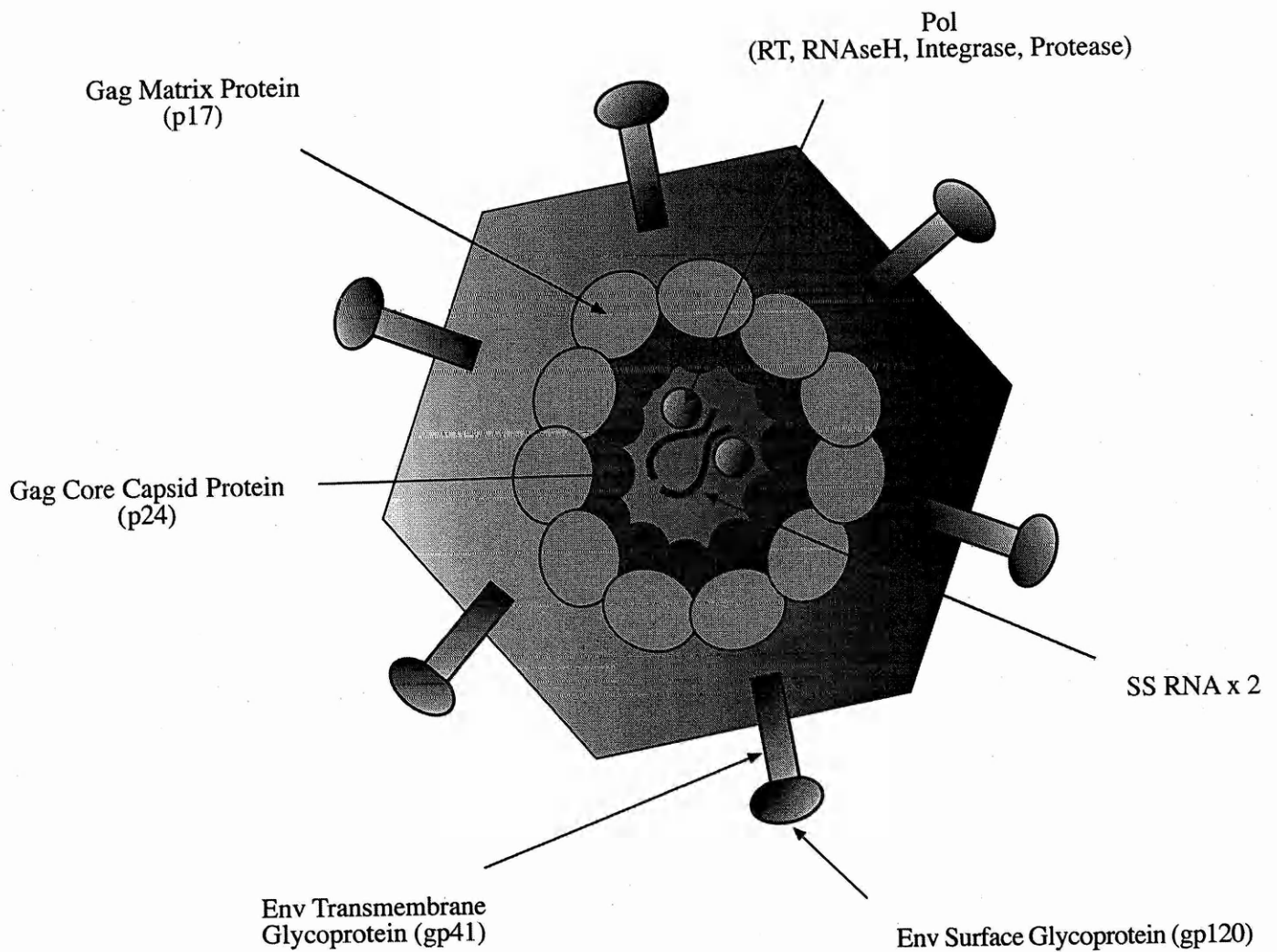
HIV-1 is a lentivirus and a member of the family Retroviridae. The general structure of the virus is presented in Figure 3. Its genome is encoded in two identical sense-strands of RNA and the virus is closely related to both HIV-2 and simian immunodeficiency virus (SIV). The sequence of cell infection and replication has been partly established. The envelope glycoprotein complex consisting of the GP120 and GP41 binds to the CD4 glycoprotein on helper T-cells (Maddon, et al. 1986, McDougal, et al. 1986). Subsequent binding of the chemokine receptor CCR5 (or in some cases, an alternate chemokine receptor) results in virus/target membrane fusion and cell entry. Different isolates of HIV use different co-receptors for cell-entry, and co-receptor usage is the principal basis for cellular tropism. HIV-IIIB and other T-cell-tropic strains of HIV-1 use the C-X-C-chemokine receptor CXCR4 (LESTR/fusin) (Feng, et al. 1996), the ligand for which, SDF-1, has recently been identified and blocks entry of T-tropic HIV isolates (Bleul, et al. 1996, Oberlin, et al. 1996). Macrophage-tropic isolates predominantly use CCR-5, a CC-chemokine-receptor which binds RANTES, MIP-1 α and MIP-1 β (Alkhatib, et al. 1996, Deng, et al. 1996, Dragic, et al. 1996) which block entry of these isolates. In some cases, HIV strains may use other members of the CC-chemokine-receptor family, principally CCR-3 (Choe, et al. 1996) and CCR2b (Doranz, et al. 1996). The crystal structure of HIV GP-41 suggests structural similarity to the influenza HA protein which plays a critical role in influenza viral fusion (Carr, et al. 1997). After entry the viral capsid is partially uncoated exposing an RNA-protein complex capable of reverse transcription. Reverse transcription is inefficient in quiescent cells suggesting the need for host components in this process (Zack, et al. 1990). Reverse transcription produces a linear double-stranded DNA, the Gag matrix protein, the accessory Vpr protein and the viral integrase (Heinzinger, 1994). The protein components are all derived from the infecting virus and no new protein synthesis is required. The pre-integration complex is transported to the nucleus where integrase catalyses the exonuclease trimming of DS-DNA, endonuclease cleavage of host

chromosomal DNA and ligation of free viral and host chromosomal DNA (Katz and Skalka 1994). The latent integrated DNA is known as the provirus. A number of viral, cellular and exogenous stimuli can activate transcription of the provirus from latency. The viral transactivator Tat can stimulate virus expression (Gaynor 1995); moreover, transcription can be initiated by mitogens, DNA damage as well as by transactivators from other co-infecting viruses (Stein, et al. 1989, Walker, et al. 1992, Zack, et al. 1988). The full length 9 Kb HIV transcript may then be used as substrate for splicing reactions giving rise to smaller spliced viral RNAs (Schwartz, et al. 1990) or it may be transported to the cytoplasm to serve as a template for synthesis of further Gag and Gag-Pol products. The process of virus assembly at the cell surface is enhanced by the Vpu protein (Jabbar 1995). The components of the virus particle, Gag and Gag-Pol, processed envelope proteins, viral RNA and Vpr protein interact at the cell surface and bud from the cell to form an immature noninfectious virus particle. Maturation of the free virus particle is mediated by the viral protease, resulting in proteolytic processing of the Gag and Gag-Pol precursor proteins and infectious virus particles (Craven, et al. 1991).

Figure 3: Structure of HIV

The major structural and functional elements of the HIV virion include the envelope (Env) glycoproteins gp41 and gp120, the Gag matrix proteins, p17 and p24, reverse transcriptase (RT), integrase, protease, and two strands of single-stranded RNA (SS RNA). The gp120 glycoprotein is the viral receptor for human CD4 while gp41 assists in viral penetration of host cells.

Figure 3: HIV Virion Structure



The role of HIV-specific CTL in the natural history of HIV infection.

There is considerable evidence that CTL play an important role in the control of HIV infection by direct effects on viral replication. This was first shown by culturing HIV from mononuclear cells derived from healthy seropositive subjects following the depletion of the CD8+ cells fraction and then suppressing virus production in a dose-dependent manner by restoring the depleted cells (Walker, et al. 1986). Further studies demonstrated that this anti-HIV activity is correlated with the patient's clinical state and in particular, with the CD4+ cell count of the infected individual (Mackewicz, et al. 1991). Particularly vigorous CTL activity has been described in a group of long-term non-progressors (Cao, et al. 1995), suggesting that this may be one of the more important mechanisms controlling the length of the asymptomatic period in HIV infection.

Although immunological abnormalities, particularly of CD4+ cell function, may be detected even in the earliest stages of HIV infection, there is nevertheless a vigorous immune response to the virus. Antibodies are generated against all the structural and nonstructural gene products, some of which neutralise heterologous isolates of HIV and others which can initiate antibody-dependent cellular cytotoxicity *in vitro*. A vigorous CTL response against HIV is also observed. The detection of HIV-specific CTL was first described in 1987 and was remarkable in that HLA-restricted CTL specific for both Gag and Env gene products could be readily detected by placing freshly separated peripheral blood mononuclear cells (Walker, et al. 1987) or in alveolar lymphocytes from patients with HIV-related pneumonitis (Plata, et al. 1987) into Cr-release assays, without any need for prior *in vitro* culture or restimulation. This level of CTL activity was previously undescribed for other virus infections and has only been detected since in infection with HTLV-1, another retroviral infection (Jacobson, et al. 1990, Parker, et al. 1992). Subsequent studies have estimated that between 15% and 88% of HIV-infected subjects have “fresh” or primary HIV-specific CTL (Grant, et al. 1992, Koup, et al. 1989, Riviere, et al. 1989, Walker, et al. 1987). The large range of detection may depend on the different assay conditions used in different labs.

MHC Class I restricted CTL have been demonstrated against most of the HIV gene products, principally Gag, Pol and Env, but also against the accessory proteins such as Nef, Tat and Vif (McMichael and Walker 1994). CTL have usually been obtained from peripheral blood, but they have also been isolated from infected organs, such as lungs (Plata, et al. 1987), lymph nodes (Hadida, et al. 1995, Langlade-Demoyen, et al. 1988), spleen (Cheynier, et al. 1994), central nervous system (CNS) (Jassoy, et al. 1992, Kalams and Walker 1995) and from the vaginal mucosa of SIV-infected macaques (Lohman, et al. 1995). Although most of the descriptions of HIV-specific CTL have been in adults, it is clear that perinatally-infected children can also mount an HIV-specific CTL response, even in the first year of life (Luzuriaga, et al. 1995).

The first identified peptide epitope was an HLA-B27-restricted 15-mer in Gag (Nixon, et al. 1988), now known to be the decamer KRWIILGLNK (Rowland-Jones and McMichael 1993). Many peptide epitopes have subsequently been reported (reviewed in McMichael and Walker 1994).

A striking feature of the cellular immune response to HIV is that the HIV-specific CTL of an infected person are directed towards multiple epitopes. This differs from previously described human and murine virus infections where a dominant CTL response has usually been identified for a given MHC allele. For instance, H-2b mice focus their entire CTL response to vesicular stomatitis virus (VSV) on a nine amino acid stretch of the nucleocapsid protein (Van Bleek and Nathenson 1990). People infected with HIV, in the mid-phase of their infection, usually make CTL responses against multiple epitopes through one or several of their HLA molecules, even though one may be the dominant response. For example, at least three peptides from Gag are restricted by HLA-B8, and CTL against all three epitopes can be detected in a single donor (Phillips, et al. 1991) and one of these donors also makes a strong CTL response to an A2-restricted peptide in reverse transcriptase (McAdam, et al. 1995, Moss, et al. 1995). Another healthy donor has been found to make CTL responses to at least six different peptides from an assortment of HIV

proteins (Harrer, et al. 1996). There are exceptions including an HIV-infected haemophiliac whose entire CTL response has been directed towards a single epitope in Gag over several years (Goulder, et al. 1997, Nixon, et al. 1988). In addition, careful study of an identical twin pair who were infected at the same time with the same strain of HIV, showed dissimilar patterns of epitope recognition suggesting that random viral mutations play a role in directing the CTL response (Goulder, et al, 1997).

Suppression of HIV replication can also occur across a semi-permeable membrane or by the transfer of supernatant from CD8+ cells, suggesting that CTL release soluble factors (CD8+ anti-viral factor or CAF) which play a role in the suppression of HIV. However, there is little consensus as to whether CAF is primarily a property of MHC Class I-restricted CTL since potent suppression has been observed without any HLA matching (Brinchmann, et al. 1990, Levy, et al. 1996, Toso, et al. 1995). However, in other studies MHC Class I matching provides maximal antiviral activity and the cells which mediate the suppression have the typical phenotype of CTL (Tsubota, et al. 1989). Detailed analysis of a panel of CD8+ clones from asymptomatic HIV-infected donors showed that the majority of suppressing clones did not exhibit HIV-specific cytolytic activity, and that some specific CTL clones showed no evidence of viral suppression - although a few clones had both properties (Toso, et al. 1995). HIV-suppressing CD8+ cells have been shown to express certain cell-surface markers: DR+, CD11b- (Toso, et al. 1995), CD28+ (Landay, et al. 1993), CD29+, CD45RA-, LFA-1+ (Tsubota, et al. 1989), CD45RO+ and CD38+, but a diversity of other markers has been observed among suppressing clones, suggesting that CD8+ clones with anti-viral activity are phenotypically heterogeneous (Toso, et al. 1995).

The CC-chemokines MIP-1 α , MIP-1 β and RANTES (Cocchi, et al. 1995) produced by CD8+ T-cells are potent suppressive factors presumably because they bind to their receptor CCR-5 thus preventing HIV co-receptor usage. These chemokines appear to be produced in an antigen-specific manner (Yang, et al. 1996, Yang, et al.

1997) and their secretion is responsive to increasing antigen exposure (Price, et al. 1998). In addition, IL-16 has some suppressive activity when produced by CD8+ cells from SIV-infected African green monkeys, but with much lower potency than the CC-chemokines (Baier, et al. 1995). The HIV-suppressive activity of human IL-16 has been questioned (Mackewicz, et al. 1996), and it likely that chemokines described to date do not account for all of the CAF activity (Levy, et al. 1996). Furthermore, additional chemokines are being identified in this rapidly progressing field. For instance, macrophage derived chemokine (MDC) has been isolated from the culture supernatant of an immortalized CD8+ clone and shown to possess suppressive activity against both laboratory and wildtype HIV strains (Pal, et al. 1998).

Initial observations showed that the activity of the CC-chemokines is greatest against macrophage-tropic and primary HIV isolates, and they have little effect against laboratory-adapted strains such as HIV-IIIB/LAI (Cocchi, et al. 1995). These observations were subsequently explained by the identification of members of the chemokine receptor family as co-receptors for HIV. These findings account for CD8+ T-cell-mediated HIV-suppression by the CC-chemokines, and explain why there has not always been consistency between past studies of HIV suppression using strains of HIV which differ in their tropism and presumably also their co-receptor usage.

The relative contribution of CTL-mediated killing and the antiviral effect of chemokines and other factors produced by CD8+ cells to control of HIV infection has yet to be determined. Antiviral factors do not affect the life-span of infected cells; if there was no T-cell-mediated cytolysis and the virus was cytopathic with a $t_{1/2}$ of 2 days, the measured half-lives of infected cells would be as described (Ho, et al. 1995, Wei, et al. 1995). If the only antiviral activity of CD8+ T cells was antigen-stimulated chemokine production, viral escape by mutation of CTL epitopes (as described below) would probably be unlikely because mutant and wild-type virus released from adjacent cells would be equally susceptible to the chemokines

although it is possible that mutant virus might stimulate the release of chemokines less effectively so there could be weaker responses in the environment of mutant virions. The increasing evidence for viral escape from CTL implies that lysis is important in control of HIV but at the cost of killing CD4+ T cells and macrophages.

This conundrum may yet prove to be important in the pathogenesis of AIDS. Lysis of CD4+ cells results in poorly functional CTL. Poorly functional CTL result in less CD4+ cell lysis and higher viral loads. Ultimately, the balance of infected and uninfected CD4+ cells and the functionality of infected CD4+ cells may determine the extent of host disease. In a situation where a small minority of CD4 cells are infected with HIV, destruction of these cells is beneficial to the host. However, in advanced AIDS where a large proportion of CD4+ cells are infected, loss of remaining cells through CTL lysis may severely compromise host defense (assuming these cells can still release IL-2 in an antigen specific manner).

MHC Class I restricted HIV epitopes

When the positions of the epitopes are mapped (for example, in Nef) it is apparent that there are clusters of epitopes in certain regions of the viral proteins (Culmann, et al. 1991). The reasons are unclear but may relate to proteasomal access to these regions of the virus. On the other hand, epitopes are more evenly distributed through Gag. There are several instances of the same epitope being presented by more than one class molecule - for example the Nef peptide 73-82 contains epitopes presented by HLA-A3, A11 and B35 (the latter is an octamer, 74-81, but the peptides presented by A3 and A11 are identical) (Culmann, et al. 1989, Koenig, et al. 1990); another Nef peptide, 190-198, is presented by three HLA-A2 subtypes as well as HLA-B52 (Hadida, et al. 1995). Although generally donors respond to the epitopes in a predictable manner, indicating the strong selective influence of the HLA type, there are examples where all donors with a particular class I molecule do not respond to the same epitope: for example, donors with HLA-A*201 usually respond either to an epitope in p17 Gag or to one in Pol, but rarely to both

(McMichael and Walker 1994). These epitopes are present in different amounts at the cell-surface of HIV-infected A2-expressing cells, with the p17 peptide being more abundant (Tsomides, et al. 1994), but this does not explain why the Pol response is immunodominant for some donors. Responses to a third HLA-A2 epitope, in a highly conserved active site region of reverse transcriptase have been rarely described (Harrer, et al. 1996). In a study of CTL responses to the Gag protein, HLA type alone did not always predict the target of the response (Buseyne, et al. 1994). It is possible that mutations in the flanking regions of CTL epitopes may lead to different rates of processing, or that immune response genes other than HLA may influence immunodominant responses, but these mechanisms have yet to be demonstrated in humans. These studies underline the extent and complexity of the CTL response to HIV.

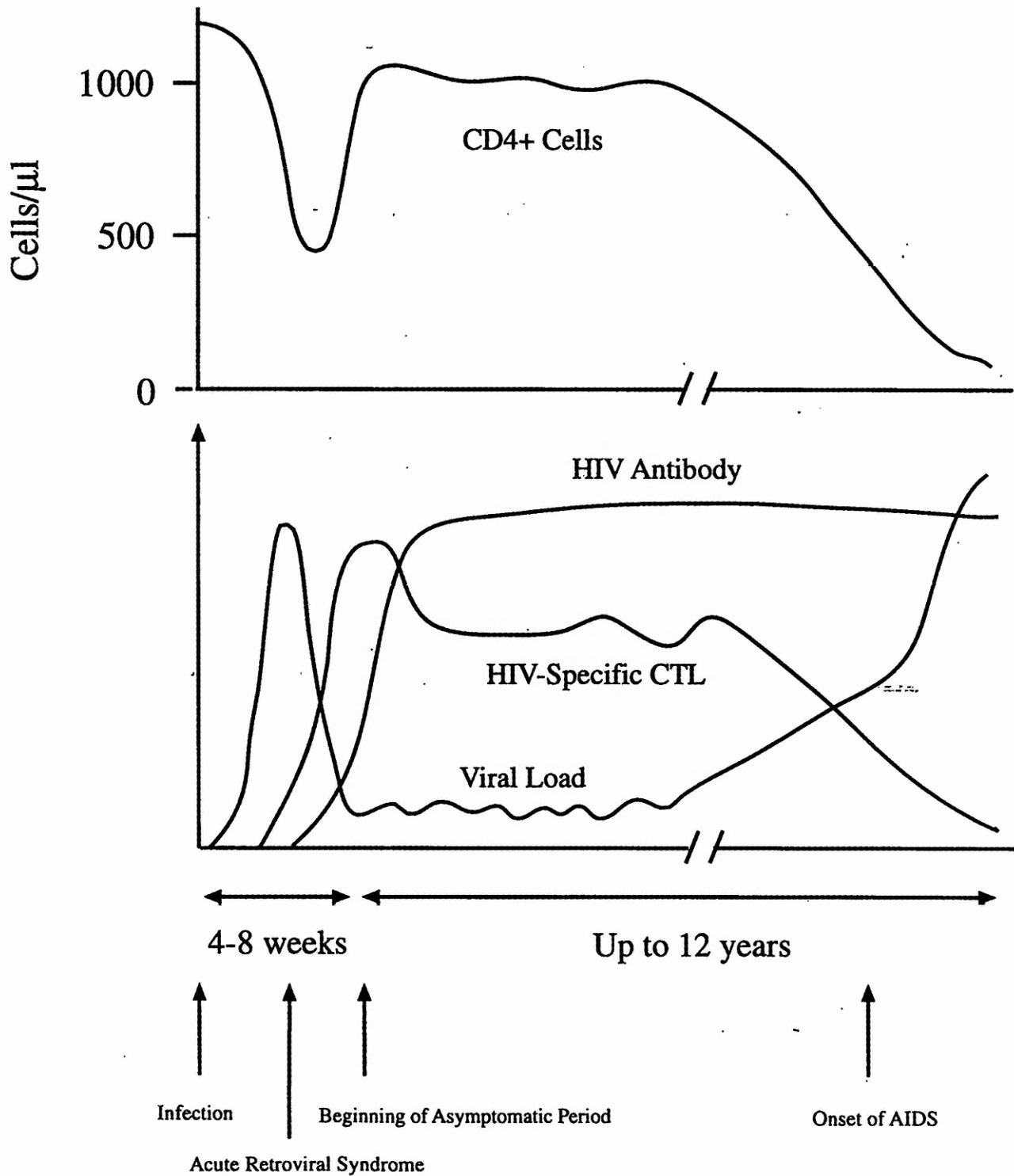
Correlation between CTL activity and disease

The natural clinical history of AIDS may be correlated to a number of laboratory parameters (Figure 4) and much of the evidence for the role of HIV-specific CTL in controlling HIV infection has come from observations of CTL activity in HIV-infected people at different stages of disease. Although correlation of CTL activity and disease state provides strong circumstantial evidence of their importance, it remains to be fully proven that CTL are directly responsible for control of virus load, rather than simply a marker of immunological good health. These studies have also attempted to be quantitative, whereas qualitative differences in CTL activity (e.g. to epitopes which cannot vary without damaging the virus) may be more important. Direct evidence that CTL are important for controlling HIV infection requires unequivocal evidence that escape mutations are selected *in vivo*, demonstration that adoptive transfer of CTL reduces virus load and demonstration that vaccine induction of CTL alone can protect against infection with HIV (or SIV).

Figure 4: Natural History of AIDS

Infection by HIV is followed at around 6 weeks by an acute viremia, manifested clinically as acute retroviral syndrome and associated with high plasma viral loads and a depressed CD4 cell count. HIV-specific CTL develop shortly thereafter followed by detectable antibody to Env glycoproteins. The viral load then decreases concomitant with a rise in the CD4 cell count and the patient is generally asymptomatic for years before a decline in both CD4 and CD8 cells and a rise in viral load signal clinical demise.

Natural History of AIDS



CTL response to acute HIV infection

CTL can be detected during early stages of acute HIV infection, even before the development of serum antibodies. The high levels of viraemia that characterise primary infection with HIV-1 and the accompanying vigorous anti-HIV immune response generally occur 3-6 weeks after HIV exposure, and are frequently accompanied by clinical symptoms of acute retroviral syndrome including fever, malaise, rash and lymphadenopathy. At this time, plasma viral RNA levels may be as high as 10 million copies per ml (Mellors, et al. 1995, Piatak, et al. 1993), and the CD4 count is low - occasionally it may be sufficiently depressed to allow the development of opportunistic infections (Gupta 1993). CD4+ T cell function is also markedly abnormal (Pedersen, et al. 1990). There is usually a profound CD8+ T-cell lymphocytosis, with huge (up to 40% of all T cells) oligoclonal expansions which express CD38, CD27 and DR but are CD28 negative (Roos, et al. 1994). In culture these CD8+CD28- cells are primed for apoptosis (Brugnoni, et al. 1996), and probably represent terminally differentiated effector CTL (Pantaleo, et al. 1994). Over the next few weeks, the plasma virus load falls by several orders of magnitude, although antibodies with the capacity to neutralise the virus are rarely detected at this stage (Ariyoshi, et al. 1992, Koup, et al. 1994).

HIV-specific CTL precursors have been described as early as two days after clinical presentation, and within three weeks of the onset of symptoms in four out of five patients in one study (Koup, et al. 1994); these CTL had specificity for the Env, Gag and Pol proteins. In another study HIV-specific CTL were detected in four out of five patients as early as 6-8 days after the development of symptoms; CTL recognising Env, Gag and Tat were generated, although the predominant response appeared to be towards gp160 (Borrow, et al. 1994). Both of these studies described single donors who failed to make detectable CTL responses and who exhibited a rapidly progressive course of HIV infection with high levels of viremia, suggesting that the early generation of a vigorous HIV-specific CTL response may not only be responsible for the initial control of viremia but also influence the subsequent disease course. Another study examining the specificity of HIV-specific CTL responses in

acute infection found that 7 out of 9 donors had detectable CTL activity in the first four weeks after seroconversion (Lamhamedi-Cherradi, et al. 1995).

Studies of the TCR repertoire in primary HIV infection have shown that the CD8+ response is represented by large but transient oligoclonal expansions in many patients (Pantaleo, et al. 1994). These expansions are marked by a large proportion of CD8+ cells bearing V β chains which have restricted amino acid sequences in the CDR3 (hypervariable) TCR region. Similar findings have been made in acute SIV infection (Chen, et al. 1995, Chen, et al. 1996). These large oligoclonal T cell expansions have also been described in infectious mononucleosis and other acute viral infections (Callan, et al. 1996, Tripp, et al. 1995). In HIV infected patients, poor prognosis was associated with a particularly narrow repertoire of CD8+ expansions, and it was suggested that a relatively limited CD8+ response may facilitate viral escape from the immune system or lead to more rapid immune exhaustion (Pantaleo, et al. 1994, Safrit and Koup 1995). More detailed study of the fine specificity of the acute HIV-specific CTL responses of two patients mapped them to epitopes in gp41; clones from these patients recognised their autologous virus sequences and continued to do so for up to 15 weeks after presentation (Safrit, et al. 1994). However, in two other patients with evidence of rapid progression, virus variants with changes in the epitopes recognised by their dominant acute CTL response, sufficient to abrogate CTL recognition, emerged during the first few months of infection (Borrow, et al. 1996, Price, et al. 1997). These last findings provide strong evidence for the importance of CTL in the control of the initial viremia.

In addition to cytolytic responses, CD8+ cell-mediated suppression of HIV replication has been described early in HIV infection, before the development of a neutralising antibody response (Mackewicz, et al. 1994). In these studies, CD8+ suppressive activity was most marked before seroconversion, and showed an inverse correlation with plasma viral load in three out of seven subjects. Further evidence to support this has come from the simian model of SIVmac infection,

where CD8+ cells capable of inhibiting SIV replication were detected within 13-60 days of experimental infection (Tsubota, et al. 1989). Finally, SIV infected macaques depleted of CD8+ cells early in infection by the use of anti-CD8 monoclonal antibodies had prolonged and high viremias as compared to mock antibody-treated monkeys (Matano, et al. 1998). Taken together, these studies demonstrate that most people with acute HIV infection develop a broadly reactive HIV-specific CTL response soon after exposure, and that the resolution of acute viraemia is in parallel with both the cytotoxic and non-cytolytic CD8+ activity.

Maintenance of the CTL response

CTL are also present during the asymptomatic phase of HIV infection. As indicated above, in the asymptomatic mid-phase of HIV infection, as many as 1% of peripheral blood mononuclear cells (PBMCs) can be effector CTL (Gotch, et al. 1990), while estimates of memory CTL range from 1 in 10^3 to 1 in 10^4 . The discrepancy between effector and memory CTL numbers is consistent with some degree of terminal differentiation of the effector CTL, possibly as a result of overstimulation: this could leave the CTL vulnerable to depletion from clonal exhaustion (Moskophidis, et al. 1993).

The vigorous CTL response against HIV is likely to result from continuous antigen stimulation by a virus that is constantly turning over in multiple sites (Ho, et al. 1995, Wei, et al. 1995). However, CTL have also been detected in long-term non-progressors with low levels of plasma viraemia (Harrer, et al. 1996). It has been theorized by Nowak and Bangham that low antigen loads might stimulate strong CTL responses if helper T cell function is good and that the same level of CTL might require far more antigen when helper T cell functions (or other accessory factors) are impaired (Nowak and Bangham 1996).

Progression to AIDS

HIV disease progresses despite the presence of virus-specific CTL. There have been several attempts to correlate either the specificity or the magnitude of the HIV-

specific CTL response with clinical outcome. Studies in the Amsterdam cohort of HIV-infected donors recruited since 1984 compared the Gag-specific CTL precursor (CTLp) frequency in long-term asymptomatic (LTA) donors and rapid progressors (Klein, et al. 1995). All of the LTA subjects had detectable CTLp against Gag, estimated at between 1/300 and 1/21,000, which were maintained over several years of follow-up, during which CD4+ cell numbers and function remained stable and virus load was low. In contrast, one of the six rapid progressors had no detectable Gag-specific CTL, and in four others it was either transient or declined to undetectable levels with disease progression. These studies demonstrated that long-term asymptomatic HIV infection is characterized by sustained Gag-specific CTL responses, although the rapid progressors were not protected from disease despite initially high levels of circulating CTL.

Studies in a large cohort (MACS) of homosexual men in Pittsburgh examined the presence of “fresh” effector CTL (CTLe) in healthy HIV-infected subjects, and detected CTLe against at least one of Gag, Pol and Env in 83% of the men during the first 8 years after seroconversion (Rinaldo, et al. 1995). There was no correlation between the levels of CTLe activity and the CD4+ or CD8+ count, or the duration of infection or the use of antiretrovirals, nor did the presence or absence of CTLe predict the disease course. These findings contrast with those in a French study, where “fresh” effector Gag-specific CTL were elicited in 18 out of 38 patients. The risk of progression to CDC stage IV disease was estimated to be 1.89 in those without CTLe to Gag, compared with those with a detectable response (Riviere, et al. 1995). There was no significant difference in the risk of progression for those with or without CTLe towards Env. These studies may be adversely affected by the insensitivity of the current CTLe assay; detectable lysis at four hours needs a CTLe frequency of around 1%; lower levels down to 0.1%, which are still high in conventional terms may be missed unless the assay is prolonged or a novel method is employed.

The determinants of disease progression are still poorly defined, complex and highly interdependent. Loss of CD4 T cells is likely to be a contributing factor (see below) and the rate of CD4+ cell loss may well be determined by virus load which is in turn is controlled by the CTL response. However, the inter-relationship between these parameters is complex (Nowak and Bangham 1996). Both virus and CTL destroy HIV-infected cells, but strong CTL responses could substantially reduce virus replication, and hence the rate of disease progression.

HIV-specific CTL decline in late stage infection

Most investigators agree that HIV-specific CTL activity becomes progressively harder to detect as disease progresses (Klein, et al. 1995, McMichael and Walker 1994, Rinaldo, et al. 1995, Wolinsky, et al. 1996). In an important study, Carmichael et al (Carmichael, et al. 1993) showed that while EBV specific CTL were still present in late disease, there was a corresponding decline in HIV-specific responses. Another recent study, in which circulating HIV-specific CTL were directly quantified *ex vivo*, Ogg et al demonstrated a significant inverse correlation was observed between HIV-specific CTL frequency and plasma RNA viral load (Ogg, et al. 1998). The HLA-peptide tetrameric complexes used in this study provide a significant technical advance in enumerating antigen specific CTL (Schwartz 1998) and will help answer further questions as to the timing of CTL loss in the latter stages of HIV infection. The mechanism by which CTL are lost in the progression of disease is critical to the success of CTL adoptive transfer experiments. Unfortunately, there are only competing theories as to why CTL should decline but little hard evidence to show that loss of CTL precedes or follows the rise in viral load and decline in CD4 cells or how this occurs.

Is the decline in CTL activity secondary to loss of CD4+ T cell help?

The most important potential mechanism is that the loss of CTL activity is secondary to the loss of CD4+ T-cell numbers and impairment of their function. It is well established that HIV infection depletes CD4+ T cells (reviewed in (Fauci 1993, Fauci, et al. 1996, Pantaleo and Fauci 1995)). If the CTL response is dependent on

Th activity, then the decline in CTL activity is inevitable if the virus is not completely controlled, and is likely to accelerate as CD4+ T-cell function deteriorates. If, as argued above, the CTL are largely responsible for the loss of CD4+ T-cells in slowly progressing patients, then ironically, CTL are responsible for their own death.

In conventional anti-viral CTL assays *in vitro*, the initial reactivation of human memory CD8+ T cells requires antigen and CD4+ T cells (Biddison, et al. 1981), and is facilitated by addition of IL-2 and IL-7 (Dong, et al. 1996, Lalvani, et al. 1994). In limiting dilution assays, the benefits of these additions are evident (Carmichael, et al. 1993). The long term maintenance of CTL *in vitro* requires, as a minimum, IL-2 and peptide antigen (de Vries and Spits 1984, McMichael, et al. 1986, McMichael, et al. 1988, Wallace, et al. 1982, Wallace, et al. 1982) and although human CTL clones can be grown in the presence of recombinant IL-2 as the only added cytokine (McMichael, et al. 1988), supernatants of activated T cells are better and imply that other cytokines are needed for optimal "help" (Wallace, et al. 1982). Although these are most likely to come from CD4+ T cells, there are other possible sources including the CTL themselves, B lymphocytes and dendritic cells.

Some experimental data, however, suggest that primary CTL responses *in vivo* may be less dependent on CD4+ "help". Lightman *et al* showed that when mice were depleted of CD4+ T cells by infusion of an anti-CD4 antibody, and infected with influenza virus, humoral responses were abolished but CTL responses remained and the mice could clear the acute infection (Lightman, et al. 1987). In LCMV infection in mice, depletion of CD4+ T cells by antibody treatment did not impair the primary CTL response, but the mice failed to maintain CTL memory during persistent infection (Matloubian, et al. 1994). However, under more rigorous conditions, Ewing *et al* (Ewing, et al. 1994) showed that in mice transgenic for an irrelevant TCR V-beta chain, although depletion of CD4+ T-cells *in vivo* greatly impaired the anti-Sendai CTL response, it had less effect on an anti-influenza CTL response and

even more convincing support for the helper T cell-independence of the primary CTL response, comes from experiments in CD4 $-/-$ (knockout) mice (Battegay, et al. 1994, von Herrath, et al. 1996) and MHC II $-/-$ mice (Hou, et al. 1995, Rock and Clark 1996) which have increased susceptibility to viral infections.

When CTL are primed with peptide epitopes, a consistent finding is that it is very difficult to prime with only class I restricted epitopes and Class II epitopes must be added (Sauzet, et al. 1995, Shirai, et al. 1996, Shirai, et al. 1994). As well, peptide priming of CTL responses in mice can be blocked by anti-CD4 treatment *in vivo* further suggestion Th cell dependence (Fayolle, et al. 1991, Gao, et al. 1991). One explanation for these seemingly contradictory data comes from experiments on the roles of antigen dose and of dendritic cells in priming of CTL. Rock *et al* (Rock and Clark 1996) primed mice with particulate ovalbumin; MHC class II presentation was required at low antigen doses, but not at higher doses. Therefore, viruses might appear CD4+ T-cell independent because the amount of antigen is usually high, whilst antigens such as minor transplantation antigens (Hurme, et al. 1978) and some allo MHC-responses (Lee, et al. 1994) might depend more on CD4+ T-cell helper for induction of CTL responses. It is probably relevant that dendritic cells, which are capable of presenting particulate antigens by the class I pathway, are sufficient to induce primary CTL responses *in vitro* without T-cell help (Bhardwaj, et al. 1994, Young and Steinman 1990).

It should be noted that dendritic cells themselves may be infected by HIV, thus acquiring the ability to process and cross-present Class I epitopes and this ability to present epitopes is not dependent upon their productive infection (Cameron, et al. 1992, Patterson and Knight 1987).

In summary, the experiments in mice indicate that priming with peptides or low dose antigen requires T-cell help, but that priming by virus infection may not. However, it has been shown repeatedly that the maintenance of CTL memory is dependent on the presence of CD4 + T cells; this could be crucial to our understanding of AIDS pathogenesis.

Is there a Th1 to Th2 switch during progression to AIDS?

A switch in the phenotype of CD4+ cells may also account for loss of help function. In 1986, Mosmann and Coffman showed that a panel of murine CD4+ T-cell clones could be classified according to their cytokine secretion profiles (Mosmann, et al. 1986). Th1 cells were characterized by the production of IL-2, IFN- γ and TNF- β while Th2 cells predominantly secreted IL-4, IL-5, IL-10 and IL-13. These secretion patterns were subsequently termed Type 1 and Type 2 responses (Clerici and Shearer 1994). Not all T-cell clones exhibit such a clear dichotomy of cytokine production, and lymphocytes which produced a mixture of Type 1 and Type 2 responses are referred to as Th0 (Street, et al. 1990). Subsequent reports have confirmed the presence of Th1 and Th2 phenotypes in human cells (but with a strong preponderance of Th0 over Th2) and defined their responses to many natural infections (reviewed in Romagnani 1994). Type 1 responses promote cellular immunity while Type 2 responses bias the immune response towards antibody production.

Infection with HIV provokes both cellular and humoral responses *in vivo*. However neutralising antibodies appear very late in the infection (Moore, et al. 1994) and seem to be relatively ineffective because of virus envelope variation. Antibodies to other virus proteins that do not neutralise are made earlier but play an ill-defined anti-viral role. As discussed above, CTL are probably the key factor in control of disease. Clerici and Shearer first suggested that a switch from a predominantly Type 1 response to a Type 2 response, resulting in decreased cellular immunity, may underlie the immune dysfunction in AIDS (Clerici and Shearer 1993). Their hypothesis was followed by some evidence that over the course of HIV infection PHA-stimulated peripheral blood lymphocytes produced increasing amounts of IL-4 and IL-10 (Clerici, et al. 1993, Clerici, et al. 1994, Meyaard, et al. 1994). Moreover, the anti-HIV suppressive factor produced by the CD8+ cells of long-term survivors as described by Levy *et al* (Levy 1993, Mackewicz, et al. 1991) appears to be dependent on a Type 1 cytokine environment (Barker, et al. 1995). It is not known

whether production of C-C chemokines is Th1 or Th2 dependent though it is clear that CD4+ clones can make them (Dong *et al*, unpublished observations).

The Th1-2 cytokine switch has not been easily found by other groups. In particular, Graziosi *et al* looked at cytokine mRNA expression in the lymph nodes of HIV seropositive patients at differing stages of disease and found little change in secretion patterns over the course of disease (Graziosi, et al. 1994). Similarly, Maggi and Romagnani were unable to show an increase in Type 2 responses in infected versus uninfected patients using PHA or PMA plus anti-CD3 stimulated PBMC (Maggi, et al. 1994). They did however find a shift towards IL-4 secretion among CD8+ T-cell clones derived from the skin (Maggi, et al. 1994), although the relevance of this is unknown.

Maggi *et al* (Maggi, et al. 1994) and Vyakarnam *et al* (Vyakarnam, et al. 1995) have demonstrated that HIV preferentially replicates in the Th2 or Th0 cells, whilst Th1 cells appear relatively resistant to infection. This correlates with evidence that virus-induced cell death is primarily of Th2 cells whereas the Th1 subset appears protected (Clerici, et al. 1994, Estaquier, et al. 1995). This could mean that a switch to an increased number of Th2 cells during infection could result in upregulated virus production in CD4+ lymphocytes and subsequent apoptosis. This hypothesis is supported by the finding that HIV infected patients with high circulating IgE levels, presumably as a result of Th2 activity, appear to have a worse prognosis (Israel Biet, et al. 1992, Lucey, et al. 1990), although in some cases this does not correlate with increased IL-4 levels (Vigano, et al. 1995). Thus patients whose immune systems are activated by atopic conditions or infections which upregulate Th2 responses may cope poorly with HIV infection, although further evidence is needed.

A complicating factor is that *in vitro* experiments do not take into account the complex cellular milieu *in vivo*. A number of other cells, principally B-cells and macrophages, also secrete cytokines which modify lymphocyte responses. Macrophages are an important reservoir of HIV infection and whereas IL-4

activates T-cells and upregulates lymphocyte virus production, the same cytokine appears has a virostatic effect upon macrophages, as do IL-10 and IL-13 (Montaner and Gordon 1995). Th2 cytokines therefore might not be wholly bad in HIV infection. If the Th2/Th0 subset of cells is preferentially infected and killed, the loss would result in lower concentrations of IL-4 in the lymphatic microenvironment and the reactivation and secretion of new virus from macrophages to renew the cycle (Montaner and Gordon 1995).

It is currently difficult to interpret whether infection and death of subsets of CD4+ lymphocytes is a result of, or the reason for, a Th1 to Th2 switch - if indeed such a switch is real. Experiments done to date appear to present conflicting data with respect to a cytokine switch during the course of HIV infection. These disagreements illustrate the complexity of the field which is highly dependent on the sensitivity and reproducibility of different protocols, on the different sampling times and specimens and perhaps even on statistical error (reviewed in (Romagnani, et al. 1994)). More convincing is the evidence that HIV replicates preferentially in Th2/0 cells and that the Th2 subset are more susceptible to apoptosis than Th1 cells. However the relevance of this phenomenon *in vivo* is still unknown.

Do CTL become exhausted?

Lymphocytes, including CTL become exhausted as a consequence of prolonged high-level stimulation. An analogous situation has been described in an animal model transgenic for an LCMV-specific T-cell receptor challenged with high doses of LCMV (Moskophidis, et al. 1993), but this was an acute phase response and there is no evidence that it occurs in humans. High levels of HIV-specific effector cells expressing the same HIV-specific TCR have been detected in peripheral blood over several years in two haemophiliac donors (Moss, et al. 1995), but the frequency of these CTL during progression to AIDS has not been studied. Recent studies of telomere length as an indication of replicative potential have shown that the CD8+ cells of HIV-infected people have significant telomere shortening (Effros, et al.

1996). This is in contrast to the CD4+ subset (Wolthers, et al. 1996), suggesting that there is much greater CD8+ cell turnover and hence potential for exhaustion.

If CTL exhaustion does occur, then failure to generate new CD8+ CTL could be a result of dendritic cell infection or infection of CD4+ CD8+ CTL precursors in the thymus. However, in a recent trial of adoptive immunotherapy using HIV-specific CTL clones transduced with marker and “suicide” genes, HIV-infected patients with CD4+ counts between 200 and 400/ μ l were able to generate CTL specific for the foreign genes and eliminate the infused CTL (Riddell, et al. 1996), so at this level of immunosuppression effective CTL responses to new antigens can still be made.

Can CTL be infected by HIV?

Despite the lack of CD4 expression, there have been occasional reports of HIV infection of CD8+ cells. For example, in long-term SIV-mac-specific CTL cultures (Tsubota, et al. 1989), and more recently in as many as 400/million CD8+ cells taken from HIV-infected people in the late stages of disease (Livingstone, et al. 1996). It is possible that HIV-specific CTL become infected at the time of lysis of infected target cells (De Maria, et al. 1991). It has also been shown in human fetal thymic explants into SCID mice that CD4+8+ thymocytes can be infected with HIV and are then depleted; as these cells are precursors of positively selected CD8+ T cells, it is possible that CTL could be infected by this route. However, this seems unlikely to be a major mechanism because multiple cell divisions are involved, and infected cells would activate virus expression and be killed by virus cytopathic effects or by the immune response.

Does HIV escape from the CTL response?

In South East Asia, the high prevalence of the HLA-A11 allele among Chinese appears to have selected a variant of EBV that has a mutation in the immunodominant epitope that it presents to CTL (de Campos-Lima, et al. 1993, de Campos-Lima, et al. 1994). If CTL are truly important in controlling HIV infection and given the variability of the virus it must be inevitable that escape mutation will

occur. The viral genome consists of 10^4 nucleotides, the mutation rate is estimated to be 10^{-5} per generation (Coffin 1995) and 10^9 - 10^{10} virions are generated every day (Ho, et al. 1995, Perelson, et al. 1996). Thus there are 10^8 - 10^9 point mutations made each day, with every possible point mutation occurring multiple times and in many combinations, although the vast majority of mutations result in defective virus. Recent evidence on the sequences of the viral proteases supports the view that the virus quasispecies contains vast numbers of mutants; protease escape mutations are there before any drug is given (Kozal, et al. 1996). Despite this enormous mutation rate, the database of HIV amino acid sequences shows that there are clear consensus sequences (Korber, et al. 1995) and nearly all patients examined have predominant virus sequences which are close to the consensus sequence (Holmes, et al. 1992, Meyerhans, et al. 1991, Phillips, et al. 1991). This implies that conserved sequences are selected at or around the time of transmission in the absence of any immune response, presumably for their transmission and survival characteristics.

Examination of envelope amino acid sequences in acute HIV infection demonstrates conservation even in the most variable part of the molecule, the V3 loop (Wolfs, et al. 1990, Zhang, et al. 1993, Zhu, et al. 1993). However, once the immune response begins to control the virus there is a diversification in the envelope sequence that must result largely if not entirely from selection by neutralising antibody (McKeating, et al. 1989, Wolfs, et al. 1990). Evidence for this comes from measurement of non-synonymous to synonymous or dN/dS (coding to non-coding) nucleotide changes in envelope sequences in macaques infected with molecular clones of SIV where certain amino acid changes were clearly selected (Burns and Desrosiers 1994, Shaper and Mullins 1993). Although antibody was almost certainly responsible, it has been suggested that there could be another force acting on the envelope (Weiss 1996). The envelope, probably the V3 region binds to a co-receptor as well as CD4 on T cells, CCR5 (or possibly CCR2b or CCR3) or CXCR4 (Alkhatib, et al. 1996, Choe, et al. 1996, Doranz, et al. 1996, Dragic, et al. 1996,

Feng, et al. 1996) and the virus could change its envelope to bind to different co-receptors and so change its tropism. It is possible that an increase in target cell range could also act as a selecting force on the envelope sequence. However, it is very likely that the high titre of neutralising antibody that is often present has a selective effect. Both antibody selection and alteration in tropism could combine as a potent force in the pathogenesis of HIV infection.

As discussed above, the initial viraemia is controlled by the CTL response: the appearance of the latter occurs just as the viraemia falls (Koup, et al. 1994). The kinetics of these grossly expanded CTL (Pantaleo, et al. 1994, Pantaleo and Fauci 1995) are almost certainly sufficient to kill most virus infected cells before new virions are produced (Klenerman, et al. 1996). Selection of escape mutants may occur at this time. Borrow *et al* (Borrow, et al. 1996) and Price *et al* (Price, et al. 1997) have both described acutely infected patients in whom there were strong CTL responses to single dominant epitopes. In both, escape mutants were selected and completely dominated the virus quasispecies within 8-10 weeks. For both examples, the mutant epitopes, one in Env and one in Nef, failed to bind to the presenting HLA molecule, HLA B44 and B8 respectively. The apparent association between oligoclonality in the primary response and poor outcome (Pantaleo and Fauci 1995) could reflect selection of such escape variants and/or over-stimulation and exhaustion of responding T cell clones.

It should be noted that unlike extracellular escape from antibody, intracellular viral escape is recessive if there are multiple virus genomes within the cell. That is, when the cell is killed all virus variants within that cell die. However as an HIV-infected cell only integrates a single copy of HIV cDNA and since most of the mutations arise pre-integration during the reverse transcription stage, it follows that all viral products in the cell should have the same mutations. It is under these circumstances that selection by CTL can occur.

In the original description of virus escape from (non-HIV) CTL, the infected mice were transgenic for an antiviral CTL TCR so that there was a monospecific selection force acting on the virus, LCMV (Aebischer, et al. 1991, Pircher, et al. 1990). Similarly, in the first trial of adoptive transfer for HIV (Koenig, et al. 1995), Koenig et al described a patient who was treated by infusion of very large numbers of a single CTL clone specific for a *nef* epitope presented by HLA-A3. This resulted in the appearance of virus with deletions in *nef* that eliminated the epitope. It is likely that the transfer of such large number of a single clone made it the dominant selection force. Under these circumstances a single mutation can evade the whole CTL response.

The descriptions of escape mutants underscore the importance of CTL in controlling the virus. They further suggest that lysis is an important *in vivo* mechanism for control of this virus infection; escape is very simple if the alternative is death. CD8+ T cells that release chemokines could also select escape mutants, but if a mutant-virus infected cell was next to a cell infected with wild-type virus, virus released from both might be equally inhibited from infecting new cells.

Do HIV-specific CTL die by apoptosis?

The proportion of CD8+ cells expressing *fas* increases during HIV infection, making them vulnerable to depletion by *fasL*-induced apoptosis (Gehri, et al. 1996). There is some evidence that CD8+ cells taken from people with late-stage HIV infection are prone to apoptosis in the presence of HIV antigens *in vitro*, which reduces the detection of HIV-specific CTL activity (Chia, et al. 1995). This is associated with reduced expression of the Bcl-2 protein which protects cells from apoptosis (Boudet, et al. 1996). Apoptosis has also been seen in the SIV models; Xu *et al* have found that many CD8+ T cells in macaques infected with a clone of SIVmac 251 showed spontaneous apoptosis, probably related to increased expression of Fas (Xu, et al. 1997). This lysis masks the CTL response because the responding CTL die in culture. In the same paper, it was shown that SIV infected cells upregulate the expression of Fas ligand. This raises the possibility that antigen-specific CTL which

respond to SIV-infected cells and which express Fas are susceptible to Fas-mediated apoptosis. This would constitute another means of viral escape outside the antigen-presentation pathway.

The mechanisms discussed above are not mutually exclusive and all could contribute to the repeatedly made observation that the CTL response that can be demonstrated *in vitro* fails as the CD4⁺ T cell level falls below 200/ml. However the relative contributions of each of these processes is important to understand because the implications for the treatment may be quite different. If the impairment of CTL activity *in vitro* is due to loss of CTL precursors, perhaps due to clonal exhaustion (Moskophidis, et al. 1993, Pantaleo, et al. 1994, Pantaleo and Fauci 1995), an effective treatment might be to replace the CTL by adoptive transfer (Koenig, et al. 1995, Riddell, et al. 1996, Riddell and Greenberg 1995) assuming that the adoptively transferred cells themselves are not exhausted. On the other hand, if it is secondary to loss of CD4⁺ T cell activity, possibly including a switch from Th1 to Th2 responses, treatment would be replacement of CD4⁺ T cells - providing a suitable donor was available (Walker, et al. 1993) or infusion of IL-2, assuming that this is the key cytokine for effecting CTL activity. Some support for this view comes from Greenberg *et al* who have treated bone marrow transplant recipients with CMV-specific CD8⁺ CTL clones (Riddell and Greenberg 1995, Riddell and Greenberg 1995, Walter, et al. 1995) They found much better survival of the infused clones when there was an ongoing anti-CMV Th response (Walter, et al. 1995).

Adoptive immunotherapy of viral infections

The adoptive transfer of autologous Class I MHC-restricted cytotoxic T-lymphocyte (CTL) clones or lines to patients infected with the human immunodeficiency virus (HIV) has been based on the success of this therapy in the control of other viral infections in humans (Riddell and Greenberg 1995) Apart from the obvious intention to treat patients, this type of study directly addresses the role of CTL in HIV infection.

In uncontrolled trials, donor derived CMV-specific CTL clone infusions protected bone marrow recipients from CMV disease until reconstitution of immune responses (Riddell, et al. 1992, Walter, et al. 1995). These studies also demonstrated the relative safety of lymphocyte infusions, the ability of transferred cells to survive up to 12 weeks in most patients and the apparent dependence of infused CTL on CMV-specific CD4+ lymphocytes for survival and proliferation. Infusion of donor leucocytes or donor derived CTL lines also appear to be effective for the treatment of EBV proliferative disorders in bone-marrow recipients (Papadopoulos, et al. 1994, Rooney, et al. 1995) and confirm the relative safety of infusions and the relative long-lived nature of infused cells.

Early experiments with adoptive transfer of lymphocytes for HIV infection have been discouraging. Ho et al attempted to augment CD8+ responses by infusing large numbers of autologous, polyclonally expanded CD8+ cells that had been obtained by leucopheresis. The protocols for expansion used only high doses of IL-2 without antigen-specific T-cell stimulation, thus failing to enrich for HIV positive cells. Such expansion protocols may be expected to aid in the CTL to a number of other infections but this was not explored. (Ho, et al. 1993). The therapy was well tolerated but significant anti-HIV effects were not observed. Other groups who have tried similar expansions and infusions have claimed improvement in opportunistic infections (Klimas, et al. 1994). Koenig *et al* transferred large numbers (around 10^{10} cells) of a *nef*-specific clone to an HIV-infected patient (CD4 count around 400/ μ l) twice over a period of 14 months (Koenig, et al. 1995). As expected the number of CD8+ cells was increased post-transfusion but surprisingly a matching rise in CTL specific lysis was not seen. This is probably because the patient still possessed high baseline activity against the *nef* epitope or, less likely, because the infused cells rapidly distributed to the lymphatic system with the displacement of non-*nef*-specific cells into the periphery. Unexpectedly, there were transient increases in virus load following both infusions and an apparent concomitant decline in CD4+ cell counts. The first infusion was given with a massive dose of IL-2 which

probably contributed to the virus replication by activating CD4+ T cells *in vivo*. However a similar rise in virus load was observed after the second infusion without IL-2. There was one direct effect of the infusion: sequencing of viral clones from the patient revealed a selection for viruses deleting part or all of the targetted *nef* epitope. This implies that the infused CTL were active against the virus but - possibly because they were monoclonal - they were ultimately ineffective. The patient subsequently died of AIDS.

Riddell *et al* have transferred Gag-specific clones to six patients with HIV (Riddell, et al. 1996). As a safety measure cells were genetically modified to carry both the hygromycin phosphotransferase gene and the Herpes simplex virus thymidine kinase (HSV-TK) gene which could, if required, efficiently phosphorylate gancyclovir and eliminate the transfused cells. Unfortunately the infused cells appeared to express HSV-TK resulting in a Class I HLA restricted CTL response and elimination of the foreign cells following the second infusion given two weeks after the first. At least the study showed that patients with CD4+ counts of around 200/ μ l can make primary CTL responses to a novel antigen. Data on Gag-specific CTL activity or virus loads after the first infusion were not published.

From the limited data presently available therefore, adoptive transfer of CTL does not appear to provide significant benefit. Administration of two or more clones may decrease the likelihood that viral mutants arise and large doses of simultaneous IL-2 may be harmful. This raises the problem of survival of the infused CTL in the recipient; data from CMV-specific CTL transfer shows that they last longer in the presence of Th activity. This is unlikely to be present in recipients with low CD4+ T-cell counts, the group with low CTL activity who might benefit from the transfer. The success or failure of CTL infusions will depend on the critical mechanisms of viral control. Comparison of adoptive transfer between HIV and other non-HIV viral infections may not be valid. In the case of CMV or EBV, cellular immunity which normally controls latent virus is absent because of bone marrow ablation and CTL infusions replace this function until host immunity is restored. In the case of HIV

host immunity is rarely if ever restored and the primary pathology is of the immune system. Thus long-lasting effects will be needed, making the role of accessory cells and factors critical.

Other mechanisms of viral control may also render CTL infusion therapy ineffective. For example, if a population of CD8+CD28- cells are primarily selected by ex vivo expansion methods, these cells when infused may be susceptible to apoptosis in vivo or fail to function adequately (Levine, et al. 1996). A recent report from Xu et al showed that in *in vitro*, wild type SIV but not a nef mutant virus leads to an increase in FasL expression on infected cells (Xu, et al. 1997). Thus the expression of FasL may protect infected cells from CTL attack, killing viral specific CTLs in the process providing a route for escaping the immune response. If such a mechanism exists for HIV, adoptive transfer of CTL may simply not work. The capacity of CTL to proliferate in vivo is also important and may be dependent on unidentified inhibitory factors (Bell and Shand 1975) or lymph node architecture which is usually destroyed in AIDS. Moreover there may remain a pool of latently infected cells which are sequestered from CTL lysis (Chun, et al. 1995) or which express viral antigens defectively (Tsomides, et al. 1994). In these cases CTL infusions are unlikely to stem the course of disease. Finally, the mechanisms by which CTL inhibit viral replication represent a combination of antigen-triggered CTL lysis as well as antigen-triggered chemokine secretion. Therefore it may be necessary to determine whether antigen-specific clones are those which produce inhibitory chemokines.

Immune reconstitution of HIV-infected patients by adoptive transfer of syngeneic whole lymphocytes or fractionated Class II MHC-restricted CD4+ lymphocytes has also been attempted. In a report from Bex *et al* (Bex, et al. 1994), an uninfected twin was first vaccinated with recombinant envelope protein from HIV and, following demonstration of envelope-specific CTL, his peripheral blood lymphocyte population was obtained by leucopheresis and transfused into the infected twin who had an undetectable CD4+ count. No changes in the clinical, immunological or

lymphocyte parameters of the recipient were noted following the first infusion. A second infusion was performed using lymphocytes pre-incubated with HIV gp160. After the second transfer there was a increase in total lymphocyte counts (both CD4+ and CD8+ cells) and an improvement in functional proliferation assays. However, there was also an increase in viral load following both transfer. The infected twin subsequently died of unrelated causes.

In a study from Lane *et al* (Lane, et al. 1990), 16 HIV seropositive twins were treated with zidovudine combined with six infusions of peripheral blood lymphocytes obtained from the seronegative twin and bone marrow transplantation at the end of the study. After lymphocyte infusions, there was an increase in the percentage of CD4+ cells present and an increase in the number of patients with delayed-type hypersensitivity reaction to tetanus toxoid. However, there was no change in the clinical status of the patients and the immunologic changes were transient. A second study which has recently begun is an ongoing phase I/II trial designed to examine the safety and efficacy of repeated infusions of activated, ex-vivo expanded syngeneic T-lymphocytes in HIV infected twins. Some data on five patients have been collected (Clifford Lane, personal communication). In one patient there was a marked rise in plasma virus coincident with each infusion. No further data is available.

Restoration of T-cell repertoire may be possible but this has yet to be demonstrated. It seems likely that transfer of activated CD4+ lymphocytes would result in the infection of infused cells and a subsequent rise in viral load. Direct transfer of lymphocytes without IL-2 or anti-CD3 activation may represent a better alternative.

Overall, immunotherapy either of anti-HIV CTL clones or more general attempts to restore the immune system is currently unproven, expensive and laborious to carry out. At present it cannot compete with the promising reductions in virus load achieved by the newer anti-retroviral drugs. However it is quite likely that, while the drugs may control the virus in the short or medium term, there could be a place for

immunotherapy in attempting to repair greatly damaged immune systems to facilitate longer term management of patients.

CHAPTER TWO

Adoptive Transfer Of Syngeneic Lymphocytes From An HIV-Uninfected Twin To His HIV-Infected Sibling

Introduction

Bone marrow transplantation in AIDS

The transition from asymptomatic HIV infection to AIDS is usually associated with the loss of CD4+ cells and an accompanying state of immune deficiency. Attempts to reconstitute the immune system either by the administration of immune enhancing cytokines such as alpha interferon (Krown, et al. 1986, Krown, et al. 1987, Andrieu, Lane et al, Rook et al) or by the transfusion or transplantation of cells have not yet been able to prevent progression to AIDS and death (Bex, et al. 1994, Contu, et al. 1993, Davis, et al. 1983, Giri, et al. 1992, Hassett, et al. 1983, Holland, et al. 1989, Lane, et al. 1984, Lane, et al. 1990, Vilmer, et al. 1987). However, because all of the trials or case reports vary in a number of critical respects, they do not rule out the possibility that an optimal regimen of bone marrow transplantation (BMT) or cell transfer combined with potent anti-retroviral drugs could ameliorate disease.

Some encouraging results have been obtained following BMT in several patients infected with AIDS. A 41 year old HIV positive male with an immunoblastic lymphoma was treated with zidovudine, cyclophosphamide and total body irradiation prior to an allogeneic marrow transplantation (Holland, et al. 1989). Engraftment occurred on day 17 with demonstrated chimerism. Both viral culture and PCR amplification of PBMC and bone marrow cells were negative for HIV 32 days post-transplantation. However, the patient died 47 days post-transplantation due to tumour relapse. Autopsy specimens were negative for HIV by PCR.

A 25 year old woman with AIDS was treated with zidovudine, busulphan and cyclophosphamide prior to syngeneic bone marrow transplantation (Contu, et al. 1993). Engraftment occurred after 18 days and tests for HIV were negative after 30 days. A second transplant from the same donor was ineffective and the patient died from acute respiratory failure 10 months after transplantation. However, p24 antigen and PCR for Env, Gag and Pol DNA became negative 30 days after transplantation and HIV serum antibodies measured by ELISA and Western blot began to decrease after 2 months, becoming completely negative 4 months after BMT. As with the report by Holland et al, PCR on autopsy tissues (skin, lungs, brain, liver, spleen) was negative for Env, Gag, and Pol.

An unpublished case of xenotransplantation was attempted on a 38-year-old patient with AIDS (Fricker 1996). The patient was given total lymphoid irradiation (TLI) and a triple combination of anti-retroviral agents (AZT, lamivudine, and indinavir) prior to transplantation with baboon bone marrow. Despite their similarity to human cells, baboon cells cannot be infected with HIV. The graft was quickly rejected but surprisingly the patient's condition improved with a decrease in viral loads and increased CD4+ counts.

These cases and others are summarised in Table 1. Taken together, these reports suggest that aggressive anti-viral therapy combined with a conditioning program can, in certain cases, drastically reduce or eliminate virus *in vivo*. Pre-transplantation conditioning by radiation or chemotherapy appears to be helpful both in promoting engraftment of cells and in eliminating cellular stores of HIV. Certainly, total ablation of white cells would destroy nearly all of the viral reservoir; moreover, engraftment and *de novo* proliferation of lymphocytes may result in an anti-viral effect.

Adoptive transfer of syngeneic lymphocytes

Immune reconstitution of HIV-infected patients through the adoptive transfer of syngeneic lymphocytes has previously been attempted by groups at the National Institutes of Health (Lane, et al. 1990) and in the Netherlands (Bex, et al. 1994). In

Table 1: Studies of bone marrow transplantation for AIDS

| Study | CD4 per μ l | BMT type | Cell Number | Conditioning | Engraftment | Anti-viral therapy | Post-transplantation course | Outcome |
|--------------------------|-----------------|------------|---------------------------|-------------------------|-------------|--------------------|--|--|
| (Hassett et al. 1983) | NA | Allogeneic | $>10^{10}$ | No | No | None | No clinical or laboratory improvement | NA |
| (Lane et al. 1984) | < 10 | Syngeneic | $>10^{10}$ | No | NA | IL-2 | No clinical improvement CD4 increase | Died after 12 mo |
| (Holland et al. 1989) | 116 | Allogeneic | 3.7×10^8 | CY TBI | Yes | AZT | Recurrence of lymphoma PCR negative from day 41 Anti-HIV antibodies declined | Died on day 47 |
| (Lane et al. 1990) | NA | Syngeneic | $2 \times 10^{10+}$ | No | NA | AZT | No clinical or laboratory improvement | 10 Alive at 23.4 months 5 Died by 17.6 months |
| (Angelucci et al. 1990) | NA | Allogeneic | $4 \times 10^8/\text{kg}$ | busulfan CY | Yes | AZT | GVHD Infections in post-transplant course | Died on day 263 |
| (Aboulafia et al. 1991) | NA | Syngeneic | NA | etoposide BCNU CY | NA | AZT | Recurrence of lymphoma No laboratory improvement | Died on day 52 |
| (Giri et al. 1992) | NA | Allogeneic | NA | CY OKT3 TLI | Yes | none | Infections in post-transplant course | Died after 13 mo |
| (Torlontano et al. 1992) | NA | Allogeneic | NA | busulfan CY | Yes | AZT | Acute GVHD Increased CD4/CD8 | Died on day 48 |
| (Contu et al. 1993) | NA | Allogeneic | 4×10^8 | busulfan OKT3 CY | Poor | AZT, GM-CSF | GVHD PCR negative from day 30 Anti-HIV antibodies negative from 4 months | Died on day 301 |

All allogeneic transplants used bone marrow from completely matched family donor.

Abbreviations: OKT3 = anti-CD3, AZT = zidovudine, BCNU = carmustine, BMT = bone marrow transplantation, CY = cyclophosphamide, GVHD = graft-versus-host disease, IL-2 = interleukin-2, NA = not available; GM-CSF = recombinant human granulocyte-macrophage colony stimulating factor, TBI = total body irradiation, TLI = total lymphoid irradiation

the first NIH study, 16 HIV seropositive twins were treated with the anti-viral drug zidovudine combined with six infusions of peripheral blood lymphocytes obtained from seronegative twins and bone marrow transplantation. After lymphocyte infusions, there was an increase in the percentage of CD4+ cells present and an increase in the number of patients with a restoration of delayed-type hypersensitivity (DTH) reaction to tetanus toxoid. However, there was no change in the clinical status of the patients and the immunologic changes were transient. The second NIH study, which was started recently, is an ongoing phase I/II trial designed to examine the safety and efficacy of repeated infusions of activated, *ex-vivo* expanded syngeneic T-lymphocytes in HIV infected twins. Limited data on five patients has been released. In one patient there was a marked but transient rise in plasma virus after each infusion. No further published data is currently available, but personal communication from Dr. H.C. Lane, NIH, suggests that the infusions have been well-tolerated, and the patients in the study have maintained or elevated CD4+ counts following therapy, with evidence of improved CD4+ cell function.

In the study from the Netherlands, an uninfected twin was first vaccinated with recombinant envelope protein from HIV, and following the demonstration of envelope-specific CTL, his peripheral blood lymphocyte population was obtained by leucopheresis and transfused into his brother, who had a CD4+ count of zero. No changes in the clinical or laboratory status of the recipient were noted after the first infusion. A second infusion was performed using lymphocytes pre-activated with HIV envelope protein. After the second transfer there was an increase in total lymphocyte counts (in both CD4+ and CD8+ cells) and an increase in functional proliferation assays. However, there was also a marked increase in viral load following the second transfer. The infected twin subsequently died of unrelated causes.

We have attempted to restore the CD4+ and CD8+ lymphocyte subsets in an HIV-infected individual by the adoptive transfer of syngeneic lymphocytes obtained from the patient's HIV-seronegative twin. Serial lymphocyte counts suggest that the *in*

vivo expansion of CD4+ and CD8+ lymphocytes occurred. TCR V-beta repertoire analysis by spectratyping reveals a distorted CD8+ repertoire in the HIV-infected twin, which remained relatively stable despite lymphocyte proliferation.

Methods

Patients

The donor was a 34 year old white, male heterosexual, non-injecting drug user serologically negative for HIV, HBV, HCV, CMV and syphilis and seropositive for EBV and VZV. The monozygotic twinship was confirmed by microsatellite sequencing (Dr. Annette Jepson).

The recipient was a homosexual found to be HIV antibody positive in November 1985 on a routine screening test in his GU clinic. There was no history of seroconversion illness and no immunological tests were done at that stage. In 1992, he had a full medical examination which revealed generalised lymphadenopathy and oral hairy leucoplakia. His CD4+ count was 400/ μ l. When he was seen in 1995, he had oro-esophageal candida, loss of weight, low grade fever, night sweats and recurrent genital Herpes simplex infection. His CD4+ count was now 40/ μ l, a full blood count showed pancytopenia and a bone marrow biopsy confirmed *Mycobacterium avium-complex* (MAC) infection. He was treated with rifabutin, ciprofloxacin and clarythromycin for the mycobacterial infection, fluconazole for fungal prophylaxis, acyclovir for recurrent Herpes zoster infection as well as trimethoprim-sulfamethoxazole for *Pneumocystis carinii* prophylaxis. In addition, he had previously been prescribed AZT and DDC although it was unclear if he continued to take these regularly. Serological tests prior to cell transfer showed that he was HBV, HCV, and Toxoplasma gondii negative but CMV and EBV positive. At the time that this study was initiated, his CD4+ lymphocyte count was 60/ μ l although but the time of cell infusion, this had decreased to 4/ μ l and he was severely cachectic. At that time, he also had documented fever, cough, diarrhea and oral hairy leucoplakia and had laboratory confirmed infections with Candida albicans,

MAC and Herpes zoster. The patient died six months after the initial infusion. No autopsy was done.

Leucopheresis

Leucopheresis was done using a COBE Spectra Apheresis System (COBE BCT, Inc. Lakewood, Colorado). Briefly, the donor twin was anticoagulated and blood was collected through a peripheral venous line at 40-60 ml/minute into cell separator which separated blood components on the basis of density and weight. The lymphocyte fraction was retained and the non-mononuclear cell components were returned to the patient via a second peripheral vein catheter site. The mononuclear cells obtained had greater than 90% purity.

TCR repertoire by flow cytometry

The monoclonal antibodies used for TCR V-beta ($V\beta$) staining are listed in Table 2. Frozen PBMC were thawed in PBS and counted. Approximately 100,000 cells were pelleted and incubated with the primary anti-TCR antibody for 30 minutes at 4°C. The cells were washed twice in PBS/Azide and co-incubated with a secondary rabbit anti-mouse FITC conjugated antibody (DAKO) and an anti-CD8+ PE conjugated monoclonal antibody (DAKO). Two-colour flow cytometry was performed on a FACScan flow cytometer (Becton-Dickenson) and 10,000 events were collected of each staining reaction. Data was analysed using Cellquest software.

TCR repertoire by spectratyping

The primers used for PCR spectratyping are listed in Table 3 and the method of Gorski was used (Gorski, et al. 1995). For CD8+ cell analysis, cells were negatively selected with magnetic beads coated with anti-CD4+ (Dyna) and then positively selected by anti-CD8+ coated magnetic beads (Dyna). Cell separation provided >95% purity by flow cytometric analysis. Approximately 1 million PBMC were lysed in RNazol and chloroform followed by centrifugation and isopropanol precipitation. Total RNA was reverse transcribed with AMV reverse transcriptase and the resulting cDNA was diluted in ddH₂O and used for amplification. The C β 3'

Table 2: Anti-TCR V-beta antibodies used for flow cytometry:

| TCR V beta | Clone | Concentration | Source |
|------------|-----------|---------------|--------------------|
| 2 | E2.2E7.2 | 1:250 | Immunotech |
| 3 | 8F10 | 1:50 | T-Cell Diagnostics |
| 5.2/5.3 | 42/1C1 | 2 µg/ml | Boylston |
| 6.7 | OT145 | 2 µg/ml | Posnett |
| 7.1 | 3G5 | neat | McMichael |
| 8.1 | 16G8 | 2 µg/ml | Immunotech |
| 9.1 | MKB P1#10 | 1:6400 | Kanagawa |
| 12.2 | S511 | 2 µg/ml | Bigler |
| 13.1 | IMMU1222 | 2 µg/ml | Immunotech |
| 13.2 | H132 | 2 µg/ml | Marrack |
| 17 | C1 | 1:250 | Friedman |
| 20 | ELL1.4 | 1:500 | Immunotech |
| 21.3 | IG125 | 1:500 | Immunotech |
| 22 | IMMU546 | 1:500 | Immunotech |
| 23 | HUT78#7 | 1:3200 | Kanagawa |

Table 3: Oligonucleotide primers used for spectratyping

| Primer | 5' - 3' |
|--------|---------------------------------|
| CbR | CTT CTG ATG GCT CAA ACA C |
| Vb1 | CAA CAG TTC CCT GAC TTG CAC |
| Vb2 | TCA ACC ATG CAAA GCC TGA CCT |
| Vb3 | TCT AGA GAG AAG AAG GAG CGC |
| Vb4 | CAT ATG AGA GTG GAT TTG TCA TT |
| Vb5.1 | TTC AGT GAG ACA CAG AGA AAC |
| Vb5.2 | CCT AAC TAT AGC TCT GAG CTG |
| Vb6 | AGG CCT GAG GGA TCC GTC TC |
| Vb7 | CTG AAT GCC CCA ACA GCT CTC |
| Vb8 | TAC TTT AAC AAC AAC GTT CCG |
| Vb9 | AAA TCT CCA GAC AAA GCT CAC |
| Vb10 | CAA AAA CTC ATC CTG TAC CTT |
| Vb11 | ACA GTC TCC AGA ATA AGG ACG |
| Vb12 | GAC AAA GGA GAA GTC TCA GAT |
| Vb13.1 | GAC CAA GGA GAA GTC CCC AAT |
| Vb13.2 | GTT GGT GAG GGT ACA ACT GCC |
| Vb14 | TCT CGA AAA GAG AAG AGG AAT |
| Vb15 | GTC TCT CGA CAG GCA CAG GCT |
| Vb16 | GAG TCT AAA CAG GAT GAG TCC |
| Vb17 | CAC AGA TAG TAA ATG ACT TTC AG |
| Vb18 | GAG TCA GGA ATG CCA AAG GAA |
| Vb19 | CCC CAA GAA CGC ACC CTG C |
| Vb20 | TCT GAG GTG CCC CAG AAT CTC |
| Vb21 | GAT ATG AGA ATG AGG AAG CAG |
| Vb22 | TCA TTT CGT TTT ATG AAA AGA TGC |
| Vb23 | CAG AGA AGT CTG AAA TAT TCG A |

oligonucleotide primer was end-labelled with γ -P32 for one hour at 37 °C in a reaction buffer containing 20 pmoles of primer, T4 polynucleotide kinase, 1X reaction buffer and 5 μ Ci of γ -P32. PCR cycling conditions were 94°C X 5 minutes for 1 cycle, 94°C x 1 minute, 62°C x 1 minute, 72°C x 1 minute for 35 cycles and 1 cycle, 94 °C x 1 minute, 62°C x 1 minute, 72°C x 10 minutes for 1 cycle. 4 μ l of PCR product was diluted with 4 μ l of formamide stop buffer and loaded onto a 6% acrylamide gel and subjected to electrophoresis at 200V for 2 hours. The gel was dried and exposed to a phosphoro-imaging plate overnight at room temperature. The plate was scanned into a phosphorimager (Molecular Dynamics) and the bands were analysed using Image-Quant software.

Virus loads

EDTA anticoagulated peripheral blood was separated by centrifugation and plasma immediately frozen. HIV virus load was determined on plasma aliquots using an RT-PCR method (Roche Amplicor).

Results

Leucopheresis

The leucopheresis procedure was well tolerated and the donor suffered no adverse physical effects. A total of 10 billion WBC was obtained from peripheral venous blood of which 54% were CD3+ (Table 4). Thus, a total of 4.8 billion lymphocytes was obtained. The proportion of CD3+CD4+ lymphocytes was 29% and the proportion of CD3+CD8+ lymphocytes was 24%, corresponding to absolute values of approximately 2.6 billion and 2.2 billion cells respectively. The cells were infused into the recipient within 16 hours of leucopheresis. Viability of the cells less than one hour before infusion, as determined by trypan blue staining was greater than 99%.

Clinical outcome

Lymphocytes obtained by leucopheresis were resuspended in 250 mls of normal saline and infused intravenously into a peripheral vein via a pump infusion setup

Table 4: Phenotype of cells obtained for adoptive transfer by leukopheresis

| | Leukopheresis (LK) cells |
|--------------------------------------|--------------------------|
| Percentage of LK cells | % |
| Lymphocytes (%) | 67 |
| CD3+ cells (%) | 54 |
| CD4+ cells (%) | 29 |
| CD8+ cells (%) | 24 |
| Corresponding absolute values | x 10⁶ |
| White blood cells | 9000 |
| Lymphocytes | 6000 |
| CD3+ cells | 4900 |
| CD4+ cells | 2600 |
| CD8+ cells | 2200 |

Lymphocytes for adoptive transfer were obtained from an HIV-negative syngeneic donor by leukopheresis (LK cells). The total number of white blood cells was estimated by an automated cell counter (Coulter) and the absolute values of lymphocyte subsets was subsequently calculated based on the cell percentages determined by flow cytometric staining using lymphocyte markers.

over a 30 minute period. The infusion was well tolerated and the patient showed no adverse effects. There were no changes in pulse rate, respiratory rate, blood pressure or body temperature. Blood samples were drawn shortly before infusion and at 1, 2, 4, 8 and 24 hours after infusion as well as at 28 days and 45 days post-infusion. At the time of infusion the patient was afebrile although in the days preceding the infusion he had episodes of fever and diarrhea. Clinical improvement following infusion was suggested by a loss of fever for weeks following the infusion, improvement in diarrhea and normalization of platelet counts (personal communication, Dr. Rabindram, Swindon General Hospital).

Hematological, Immunological and Virological Results

Blood samples were taken according to the schedule in Table 5 and sent for routine clinical laboratory testing. A sample from the donor twin obtained prior to leucopheresis and a sample of the leucopheresis cells were also submitted. A full blood count as well as flow cytometry to determine the proportion of CD4+ and CD8+ cells was performed on the samples. Baseline hematology counts showed anemia and pancytopenia. An absolute CD4+ lymphocyte count of 4/ μ l (2% of total lymphocytes) rose to 29/ μ l (14% of total lymphocytes) one hour after infusion and then decreased thereafter. However, on day 28 the CD4+ count had risen to 120/ μ l before falling to 9/ μ l at day 45 post-infusion. The absolute CD8+ lymphocyte count also rose from a baseline value of 146/ μ l to 1220/ μ l at day 28 and by day 45 had decreased to 830/ μ l. The proportion of CD25 (IL-2Ra) positive lymphocytes did not change before or after infusion. Double staining of lymphocytes for CD4+ and CD25+ or CD8+ and CD25+ was not done.

The plasma concentration of HIV RNA was determined on the HIV-infected twin (Table 5). Plasma virus RNA levels decreased nearly one-logfold one hour post infusion from a baseline of 1.5 million copies/ml to 350,000/ml. This rose subsequently to a concentration of 1 million copies/ml at day 45.

Table 5: Hematological, immunological and virological laboratory markers before and after the adoptive transfer of syngeneic lymphocytes.

| | Pre | +1hr | +2hr | +4hr | +8hr | +24hr | + 28d | +45d |
|--|------|------|------|------|------|-------|-------|------|
| Hgb(mg/dL) | 8.5 | 8.4 | 8.9 | 9 | NA | 8.5 | 9.5 | 10 |
| WBC | 1.8 | 1.9 | 3.0 | 2.7 | 3.1 | 1.5 | 5.6 | 5.1 |
| Platelets () | 95 | 85 | 90 | NA | NA | NA | 223 | NA |
| Lymphocytes (%) | 10 | 11 | 17 | 15 | 20 | 19 | 30 | 18 |
| T-cells (%) | 90 | 86 | 90 | 90 | 82 | 87 | 82 | 96 |
| CD4 (%) | 2 | 14 | 5 | 4 | 3 | 3 | 7 | 1 |
| CD8 (%) | 81 | 67 | 80 | 80 | 77 | 83 | 72 | 91 |
| CD25 (%) | 9 | 12 | 7 | 7 | 4 | 8 | NA | 3 |
| Lymphocytes (/µl) | 180 | 209 | 510 | 405 | 650 | 285 | 1700 | 913 |
| T-cells (/µl) | 162 | 180 | 459 | 365 | 533 | 248 | 1390 | 876 |
| CD4 (/µl) | 4 | 29 | 26 | 16 | 20 | 9 | 120 | 9 |
| CD8 (/µl) | 146 | 140 | 408 | 324 | 501 | 237 | 1220 | 830 |
| Viral load (x 10 ³ copies/ml) | 1490 | 350 | 540 | 510 | 820 | 980 | NA | 1020 |

Blood samples were obtained from the HIV-positive patient before and after (from 1 hour to 45 days post-transfer) adoptive transfer of syngeneic lymphocytes. Samples were assayed for hemoglobin (Hgb), total white blood cells (WBC) and platelets by standard clinical laboratory methods. In addition, flow cytometry was performed to determine the proportion of CD3, CD4, CD8 and CD25 positive cells in each sample. Absolute values of CD3, CD4 and CD8 lymphocytes were then calculated and expressed per cubic millimetre (µl). NA = not available

Table 6: TCR V-beta families determined by flow cytometry

| Vb family | LK % | Δ SD | AF Pre % | Δ SD | AF +24h % | Δ SD | Mean % | Mean SD |
|-----------|------|-------------|----------|-------------|-----------|-------------|--------|---------|
| Vb1 | 3.5 | 0.5 | 2.6 | 0.2 | 1.8 | 0.8 | 2.79 | 1.35 |
| Vb2 | 6.6 | 0.6 | 5.3 | 0.2 | 6.6 | 0.6 | 5.52 | 1.67 |
| Vb3 | 1.9 | 1.1 | 9.8 | 2.5 | 12.8 | 3.9 | 4.33 | 2.18 |
| Vb5 | 2.9 | 0.5 | 2.8 | 0.6 | 1.6 | 2.0 | 3.25 | 0.83 |
| Vb6.7 | 2.3 | 0.4 | 1.0 | 0.6 | 0.8 | 0.8 | 1.77 | 1.34 |
| Vb7 | 0.9 | 0.9 | 0.7 | 1.2 | 0.4 | 1.5 | 1.59 | 0.79 |
| Vb8 | 4.8 | 0.4 | 1.9 | 2.7 | 2.6 | 2.0 | 4.48 | 0.94 |
| Vb9 | 3.0 | 1.3 | 1.8 | 0.6 | 1.7 | 0.8 | 2.16 | 0.63 |
| Vb11 | 1.9 | NA | 0.4 | NA | 0.5 | NA | NA | NA |
| Vb12 | 6.4 | 3.7 | 3.1 | 0.8 | 2.6 | 0.3 | 2.24 | 1.13 |
| Vb13.1 | 3.2 | 1.2 | 3.7 | 1.7 | 4.2 | 2.2 | 1.92 | 1.04 |
| Vb13.2 | 1.1 | NA | 1.0 | NA | 2.2 | NA | NA | NA |
| Vb14 | 6.7 | NA | 7.3 | NA | 7.8 | NA | NA | NA |
| Vb16 | 1.2 | NA | 1.0 | NA | 0.9 | NA | NA | NA |
| Vb17 | 3.7 | 1.6 | 1.8 | 2.5 | 1.7 | 2.6 | 6.91 | 2.05 |
| Vb18 | 0.4 | NA | 0.4 | NA | 0.1 | NA | NA | NA |
| Vb20 | 2.2 | 0.7 | 2.3 | 0.8 | 1.3 | 0.4 | 1.62 | 0.84 |
| Vb21.3 | 3.2 | 1.7 | 2.8 | 0.9 | 2.0 | 1.0 | 2.44 | 0.43 |
| Vb22 | 3.3 | 0.1 | 3.3 | 0.1 | 2.6 | 0.3 | 3.1 | 1.68 |
| Vb23 | 1.7 | NA | 1.6 | NA | 0.7 | NA | NA | NA |

Peripheral blood mononuclear cells (PBMC) were obtained from the recipient at the indicated timepoints, stained with fluorescent-labelled antibodies specific for different TCR V-beta families and quantified by flow cytometry. Values refer to the percentage of total cells staining with a particular TCR V-beta antibody. Abbreviations: Vb = V beta, LK = donor PBMC, AF Pre = recipient PBMC prior to adoptive transfer, AF +24h = recipient PBMC 24 hours after adoptive transfer and Δ SD = standard deviation. Mean % refers to the mean values obtained from ten healthy individuals. Standard deviations from the mean which are > 3 have been bolded. NA = not available

Repertoire analysis by flow cytometry

CD4+ analysis by flow cytometry was not technically possible because of an insufficient quantity of CD4+ lymphocytes present in the peripheral blood. Flow cytometric analysis of the CD8+ TCR subset was done using V-beta family specific monoclonal antibodies. A mean percentage of V-beta subsets was determined by collecting samples from ten healthy individuals. Expansions have previously been defined as percentages which fall outside 3 standard deviations from the mean as calculated by assaying the repertoires of healthy individuals (Rebai, et al. 1994). Using this definition, very few V-beta expansions or deletions were detected by monoclonal antibodies in either the CD8+ cells derived from leucopheresis of the healthy twin or from the CD8+ lymphocytes before and after infusion in the HIV+ twin (Table 6).

Repertoire analysis by spectratype

The TCR V-beta repertoire was also determined by spectratyping on the CD4+ and CD8+ subset of cells obtained from the leucopheresis cells (shown in Figure 5) and on the CD8+ subset in the recipient's peripheral blood mononuclear cells at Time 0, +24 hours and +28 days (shown in Figure 6).

Figure 5: Donor T cell repertoire determined by spectratyping

PBMC obtained from the donor by leucopheresis were purified using magnetic beads conjugated with antibodies to either CD4 or CD8. Complementary DNA (cDNA) synthesized from either the CD4+ or CD8+ lymphocyte fractions were then subjected to TCR V-beta family "hot" PCR using a forward primer specific for V-beta germline sequences and a reverse primer specific for the TCR constant region. The PCR products were then separated by acrylamide gel, exposed to a radioactivity-sensitive screen and scanned into a phosphorimager. Each different sized band within a TCR V beta family represents an amplified cDNA TCR V-beta segment with a unique variable joining and/or diversity region.

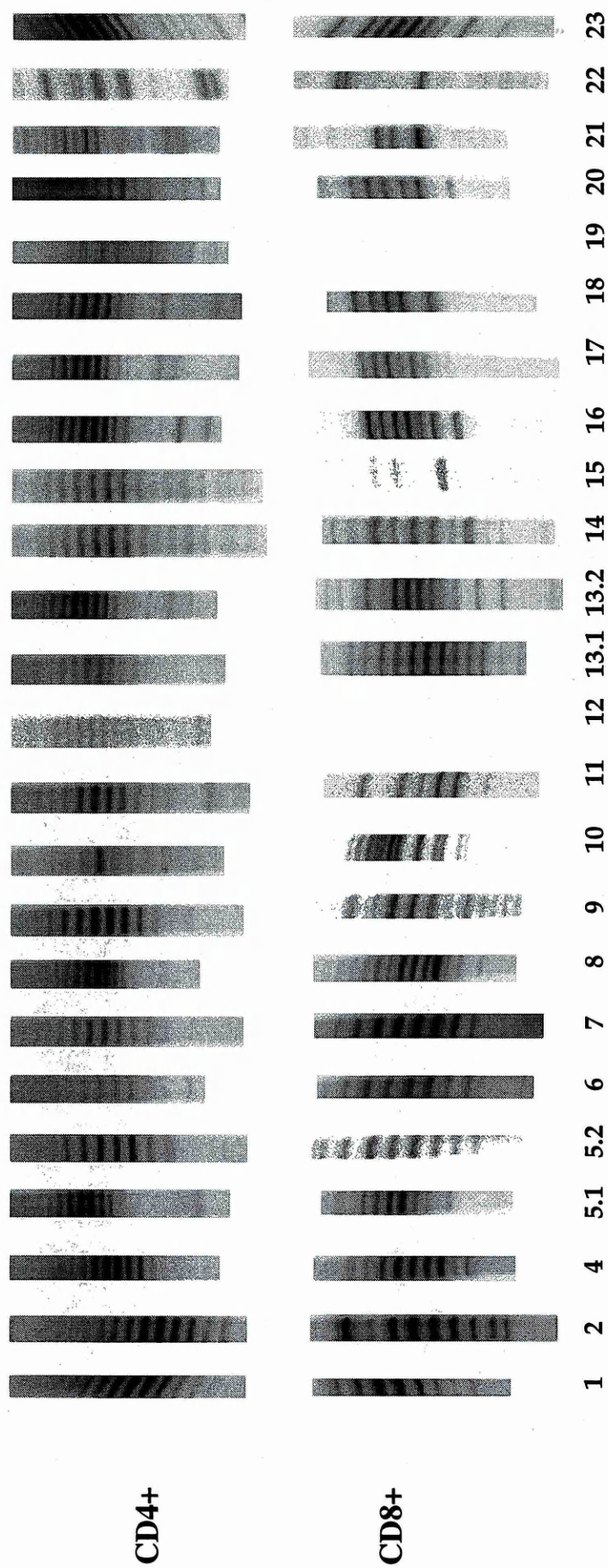


Figure 5: Donor CD4+ and CD8+ TCR V-beta repertoire by spectratype

Figure 6: Recipient CD8+ T cell repertoire determined by spectratyping

PBMC obtained from the recipient at the indicated timepoints, before and after adoptive transfer, were purified using magnetic beads conjugated with antibodies to either CD4 or CD8. There were insufficient CD4 cells to generate cDNA and only cDNA obtained from CD8 cells was subjected to spectratyping. Complementary DNA (cDNA) synthesized from either the CD8+ lymphocyte fractions was subjected to TCR V-beta family PCR as described in Figure 5. Arrowheads indicate unchanged repertoire (V-beta 2.1) and expansion of existing repertoire (V-beta 23). Asterisk indicates formation of a new repertoire.

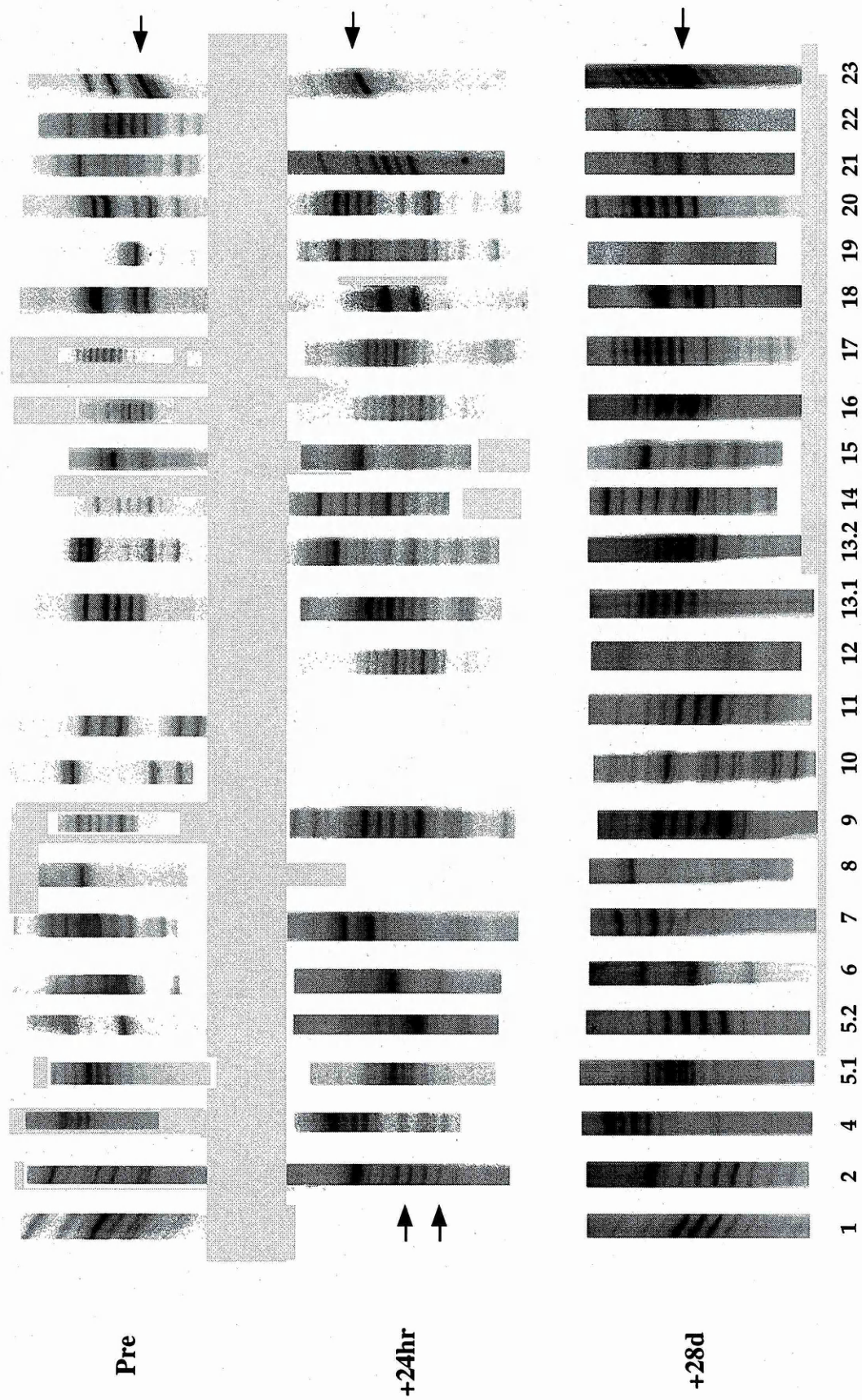


Figure 6: Recipient CD8+ TCR V-beta repertoire by spectratype

There is neither a close correlation between the CD4+ and CD8+ repertoire obtained from the same individual or perturbations in the repertoires obtained from this healthy individual. In accordance with previous observations, there is also little correlation between the TCR repertoires of the healthy donor and his HIV+ monozygotic twin (Hawes, et al. 1993, Rebai, et al. 1994).

Discussion

Lymphocyte expansion and clinical course

There were encouraging signs in our patient that the transferred syngeneic lymphocytes were actively proliferating. By the day 28 after cell transfer, the CD4+ lymphocyte count had risen from 4/ μ l to 120/ μ l and the CD8+ lymphocyte count had risen from 146/ μ l to 1220/ μ l. If these expanded cells were functional, at least a temporary benefit could be attributed to the infusions. The lymphocyte proliferation present in both subsets could be ascribed to encounters with novel or recall antigens, followed by activation and proliferation secondary to CD4+ mediated IL-2 production. For example, since the recipient was actively infected with VZV (Herpes zoster) and the donor was serologically positive for VZV, reactivation of VZV-specific CD4+ and CD8+ memory cells present in the infusion could produce an extensive proliferation of lymphocytes. The same argument can be made for EBV since the recipient was suffering from oral hairy leucoplakia and the donor was EBV seropositive. Any EBV-specific memory cells present in the infusion would be expected to undergo clonal expansion upon encountering active EBV infection. In this regard, the infused cells may have played a role in augmenting the patient's immune response to MAC. Prior to infusion, the patient (who was non-compliant with his anti-mycobacterial drug regimen), suffered from fever, chronic diarrhea and pancytopenia. These symptoms improved after the infusion and in particular, his platelet count rose from a baseline level of around 95/ μ l to 223/ μ l two months after the infusion suggesting improved production of megakaryocytes. Alternatively, the cells may have simply been repopulating depleted lymph nodes where they would be expected to undergo expansion in order to maintain lymphocyte homeostasis.

Extensive repopulation of lymph nodes by post-thymic T-cells has been demonstrated in several murine models of adoptive transfer (Rocha 1987; Miller and Stutman 1984; Bell, et al. 1987). In most cases, the repopulation falls short of normal numbers of lymphocytes, although functionally the lowered populations do not appear to result in an abnormal host immune response. Perhaps more importantly, the recipient mice in all the experiments were irradiated, depleting them not only of lymphocytes but also of other factors which may inhibit lymphocyte expansion (Bell and Shand 1975). Whether HIV-mediated destruction of lymph nodes provides a suitable environment for lymphocyte expansion is not known although the evidence is that it does not. Certainly, the increases in CD4+ cells in our patient, mirrored by a rise in CD8+ cells suggest that even if repopulation of lymph nodes was not occurring, antigen-driven responses were. Other groups who have also transferred syngeneic lymphocytes to HIV-infected recipients have reported percentage increases in CD4+ lymphocytes but no serial data to estimate immediate kinetics are available (Lane, et al. 1990; Lane, et al. 1984). Our data indicate that a small rise in CD4+ count is detectable one hour after infusion (Table 4) and that a concomitant rise in CD8+ cells is detectable at 2 hours. By 24 hours however, these counts were nearly back to baseline levels, suggesting a redistribution of cells to the lymphoid system. This redistribution of cells following adoptive transfer in mice has been documented in detail (Pape, et al. 1997). Unfortunately, our patient refused any further therapy or bloodwork beyond 45 days and neither repeated infusions nor repeated lymphocyte counts could be done.

Virus loads

The virus load inexplicably decreased nearly one logfold one hour after cell infusion, followed by a rapid increase until it was approximately 65% of the baseline level at 24 hours. It is difficult to explain this rapid decrease on the basis of CTL activity as the one hour time period is too short for any significant effector function (either killing or cytokine secretion) to occur. Similarly, the fall in virus concentration cannot be explained by a dilutional effect because of the relatively small volume of fluid (250 ml) infused. Ho et al (David Ho, personal communication) have reported

that following bolus infusions of SIV into SIV-negative and SIV-positive macaques followed by animal sacrifice, less than 2% of the viral RNA can be accounted for following autopsy. He suggests that the virus may have undergone RNA degradation following infusion. Our case is a reverse of that situation, in that instead of infusing a bolus of virus to naive recipients, we are infusing a bolus of naive lymphocytes to an individual with high concentrations of circulating virus. The large numbers of cells infused may have "mopped up" free viral particles and taken them out of the peripheral circulation temporarily. Certainly, the kinetics of viral decrease correspond with the immediate rise and fall of lymphocyte counts. RNA degradation seems less likely since the virus loads returned to 2/3 of normal values within 24 hours.

TCR repertoire

The TCR V-beta repertoire of monozygotic twins discordant for HIV infection has been examined previously. In general, most groups report that TCR repertoires in HIV infected patients vary widely in contrast with those of their healthy monozygotic twins (Rebai, et al. 1994) while the TCR of identical, uninfected twins is relatively concordant and much closer than that of unrelated individuals (Hawes, et al. 1993, Lovridge, et al. 1991). We have examined the CD8+ repertoire of the uninfected and infected twin by flow cytometry and spectratyping. By flow cytometry, few expansions could be detected in any V-beta subset. This is surprising in light of the fact that the infected twin was harbouring numerous infectious and pathogenic organisms. But on closer examination, it may not be surprising. Depletion of repertoire is common in the late stages of HIV infection and an absolute lack of CD8 cells was certainly present before and 24 hours after transfer. Spectratyping may be expected to show TCR expansions not present by flow cytometry; however, because we did not amplify any normal cellular or housekeeping genes we do not have a standard with which to compare band intensity. Moreover, since the amount of V-beta mRNA transcript present intracellularly depends on the activation state of the cells (Paillard, et al. 1990) inaccuracies are expected to occur when quantifying expansions. Thus, we can only

remark on whether the TCR V-beta families are perturbed or not perturbed and unlike repertoire determination using monoclonal antibodies, we cannot comment on the actual percentage of V-beta families present.

In agreement with other reports (Rebai, et al. 1994) we found that there was very little concordance in the CD8+ V-beta usage between the HIV-infected and uninfected twins. The uninfected twin had a full and normal spectratype repertoire with few band deletions (Figure 5). In contrast, the infected twin at the pre-infusion timepoint had a limited repertoire (as shown by the numerous missing rungs in the ladders) and oligoclonal usage of V-beta subsets. At 24 hours after cell transfer, there was little change in the repertoire although there is suggestion of an additional clonal use in the V-beta 5.2 family. Expansions and limited repertoires which were present before infusion mostly remained 24 hours after infusion suggesting that there was not time for the infused cells to undergo significant replication or that the infused cells conformed to the ongoing clonal proliferations. However, four weeks after infusion PCR expansions were still largely maintained favouring the latter hypothesis - that ongoing clonal stimulation of infused cells was continuing. In one subset, Vb5.2 a more normal repertoire had been restored.

Another method for determining expansion is to measure band intensities. Using criteria established by others (Monteiro, et al. 1995), limited TCR usage was determined in selected V-beta subsets by defining an expansion as a single band present in spectratyping which accounts for greater than 50% of the total intensity of all bands. By these criteria, expansions were present in several different subsets at several different timepoints and generally correlate with a visual interpretation of the data (Figure 6). For instance, the Vb18 and Vb23 families had limited bands present on the spectratype gel and intensity analysis confirms expansions present at all three timepoints. However, an inherent flaw in the above argument is that it is difficult to distinguish between a clonal expansion and a loss of repertoire. Lanes exhibiting only one or two bands may indeed have undergone clonal expansion (supported by the similar basepair lengths of the amplified CD3 regions) or alternatively they may

have undergone a deletion of clonal subsets leaving a restricted repertoire consisting of one or two bands. Because of our inability to quantify these PCR based results (as opposed to antibody staining and flow cytometry), it is not possible to distinguish between these two options. Moreover, because the paucity of cells obtained limited the amount of cDNA available for cloning and sequencing, it was not possible to determine whether these expansions were clonal or oligonclonal .

Imberti et al have postulated that the widespread deletions of V beta repertoire which they detected in a patient in the late stages of disease may be due to an HIV-encoded superantigen (Imberti, et al. 1991). This is not supported by studies which show random depletion of V-beta repertoire in patients with AIDS as compared to uninfected controls (Boldt Houle, et al. 1997)(Boyer, et al. 1993). In our patient, no absolute deletions were present despite a CD4+ count of less than 10/ μ l.

Exposing HIV-naïve lymphocytes to replicating virus should in theory, mimic the situation of acute infection. Pantaleo et al have shown that large expansions of particular CD8+ V-beta families occur following acute infection with HIV (Pantaleo, et al. 1994). Of the six patients studied, maximal expansions of V beta subsets occurred at various timepoints after the onset of symptoms ranging from day 6 in one patient (where 18% of the PBMC analysed belonged to the V beta 4 family) to day 65 in another patient (where 12 % of the PBMC belonged to the V beta 8 family). Acute infection with EBV also drives large antigen-driven clonal CD8+ expansions are present in acute infectious mononucleosis (Callan, et al. 1996). Thus, in our patient, exposure of donor lymphocytes to antigens derived from infection with M. avium-complex, H. simplex and Varicella zoster should have induced expansions in the relative V-beta families within the CD8+ subset as has been described for other infections (Islam, et al. 1996). Exceptionally high TCR V gene usage has been described in many infections as well as in healthy individuals making interpretation of T-cell clonal expansions difficult.

Restoration of repertoire may be a key event in the treatment of AIDS. Despite the rebound increase in CD4+ cell counts following use of antiretroviral therapy (in particular, therapy which uses at least one protease inhibitor) a number of early reports suggest that susceptibility to opportunistic infections remains high. In a series of 210 patients, 10 cases of acute CMV infection occurred within 10 weeks of initiating indinavir or zidovudine plus RT analogs (Poster Abstract 354, 4th Conference on Retroviruses and Opportunistic Infection). This occurred despite a greater than five-fold increase in CD4+ cell counts (from 32 +/- 27/ μ l to 150 +/- 107/ μ l). Similarly, Michelet (Poster abstract 315, 4th Conference on Retroviruses and Opportunistic Infection) described 5 cases of CMV retinitis and 1 case of VZV retinitis among 80 patients shortly after they started on HAART. Jacobson et al reported 5 HIV-seropositive patients with baseline CD4+ counts of less than 85/ μ l who, following antiretroviral treatment, had a rise in CD4+ counts to greater than 200/ μ l within 4-8 weeks, yet soon thereafter developed CMV retinitis (Jacobson, et al. 1997). Using PCR spectratyping, the CD4+ TCR repertoires were examined before and after antiviral or antiviral plus IL-2 treatment (Connors, et al. 1997). Disruptions of the normal size patterns were observed in all 11 HIV-positive patients treated and these were associated with depletions in TCR-beta families. Abnormalities were most prevalent in patients with the lowest CD4+ T-cell counts. Both IL-2 and protease inhibitor therapies increased CD4+ counts but led to minor, if any changes, in disrupted TCR families. Thus the problem remains that naive T-cells are not being restored by anti-retroviral therapy. Paradoxically then, antiretroviral treatments may be most effective in patients prior to clinical disease when TCR repertoires may be relatively undisturbed. Thus the goal would be the early protection of T cell repertoire rather than the "unobtainable" restoration of T cell repertoire.

If highly active antiviral agents can decrease virus loads several fold and restore CD4+ and CD8+ lymphocyte subsets, then this is clearly the therapy of choice for those who have access to it. However, the limited data present suggests that the loss of TCR repertoire in late disease cannot be replaced by naive lymphocytes or stem cells. Restoration of global CD4+ counts is still unable to protect some individuals

from opportunistic infections. Similarly, there are individuals with extremely low CD4+ counts who remain free from opportunistic infection. These individuals may represent the flip side of the repertoire analysis - that is, despite a low overall CD4+ count, the CD4+ lymphocytes which are present represent a large range of protective V-beta families. If this is the case, then treatment with highly active drugs should be instituted early in disease when the TCR-repertoires are relatively intact. For those individuals who have lost parts of their CD4+ or CD8+ repertoire, lymphocyte infusion may still be an option.

Outlook for lymphocyte transfer and BMT in AIDS

Ideally, the transfer of syngeneic lymphocytes would result in their engraftment and expansion. This is dependent on the ability of the host lymph nodes to permit expansion and this in turn, is probably dependent on pre-conditioning the patient with immunoablative therapy. In this respect, the previously published case reports on the use of BMT in patients with AIDS are extremely instructive. Although all these cases ultimately had undesirable outcomes, it is encouraging that in at least two patients, the virus appeared to be eradicated as demonstrated by reversal of serological status and negative PCR of autopsy tissues. In addition, both patients had marrow engraftment as was demonstrated by complete chimerism although this may simply reflect the fact that both patients were given conditioning therapy. Although rare case of spontaneous clearance of HIV have been reported (Burger, et al. 1985, Farzadegan, et al. 1988, Roques, et al. 1995) it seems unlikely that they would account for the disappearance of virus in this setting particularly since those cases usually involved patients with low virus loads and high CD4+ counts. cursory examination of the data in Table 1 suggests that pre-transplantation conditioning may benefit patients undergoing transplantation. As was argued with adoptive therapy, prior destruction of myeloid and lymphoid cells by irradiation or chemotherapy appears to be necessary for optimal engraftment of marrow or expansion of lymphocytes either by creating "space" or more likely, by removing cells which interfere with engraftment and expansion (Bell and Shand 1975). Equally important however, is that the cells most affected by ablative therapy are

the same cells which act as primary reservoirs for HIV. Thus, massive destruction of the hematological and immunological subsets of cells would result in a dramatic decrease in virus load. Such destruction may remove reservoirs of long-lived virus whose half-life of clearance by protease inhibitors is prolonged (Chun, et al. 1997, Perelson, et al. 1996). Ablative conditioning combined with highly active antiretroviral therapy may therefore result in near total elimination of the virus. This question was not answered by previous case reports because the majority occurred before the advent of protease inhibitors. Thus, sub-optimal anti-viral therapy (consisting of a single reverse transcriptase inhibitor) generally was used. In the case of the baboon transplant where despite graft rejection, the patient improved, triple therapy involving indinavir was used.

In the case of lymphocyte transfer, the quality of cells given to infected patients is also important. Levine et al have reported the growth of a population of CD4+ cells resistant to infection with NSI strains of HIV (Levine, et al. 1996). The combination of anti-CD3 and anti-CD28 (and not PHA and IL-2) produces a complete down-regulation of CCR5 in the stimulated cell population (Carroll, et al. 1997). At the same time, CXCR4 expression increases significantly, and the cells are highly infectable by T-cell-tropic virus. It is not known how long this effect will be maintained, but the previous studies of this group showed that the CD4+ cells activated in this way were resistant to HIV infection for several weeks. It would be interesting to transfer these *in vitro* resistant cells to patients with advanced immunodeficiency. However a number of known problems needs to be addressed. If a loss of the CD4+ TCR repertoire underlies the pathogenesis of immune deficiency, the expanded cells will need to express a full range of TCR V-beta families; the polyclonal nature of the CD3/CD28 stimulation cell expansion has been shown to provide this. It will also be important to examine Fas expression on the expanded cells before infusion, to avoid their rapid elimination by apoptosis when they encounter HIV-infected cells with high Fas-L expression (Xu, et al. 1997). Cells generated by costimulation with anti-CD3/CD28 do however appear to be resistant to apoptosis (Levine, et al. 1996) and are also less susceptible *in vitro* to the adverse

effects of gp120 on proliferation and cytokine secretion (Faith, et al. 1996). It has also been observed that there is progressive down-regulation of CD28 during the course of HIV-infection - these cells may be refractory to the CD3/CD28 stimulation regime (Borthwick, et al. 1994). Most importantly however, cells expanded using the above protocol produce cells with upregulated CXCR4 expression. The impact of this cell phenotype on virus evolution is not known. However, homozygotes for the D32 mutation in CCR5 do not seem to be protected from rapid disease progression if unlucky enough to become infected (Biti, et al. 1997, O'Brien, et al. 1997, Theodorou, et al. 1997). In most patients with late-stage disease, SI variants predominate, so the rationale for transfer of activated cells with maximal CXCR4 expression is poor. Ironically, it may be better to transfer cells to patients who have not yet developed AIDS, but in whom rising viral load and CD4+ cell decline foretell impending immunodeficiency. This approach still has potential, but until more information is available about the key mechanisms underlying CD4+ cell depletion, the potential consequences of cell transfer for virus evolution cannot be completely evaluated.

In summary, we have demonstrated that polyclonal expansions in CD4+ and CD8+ lymphocytes can occur after syngeneic lymphocyte transfer. These expansions appear to be maximal around 28 days after infusion, in keeping with the timeframe present in stem cell transplantation in which bone marrow repopulates around 5 weeks. Clinical correlation with the expanded lymphocytes is anecdotal although platelet counts returned to normal. This may have represented a restored lymphocyte response to his opportunistic pathogens but definitive proof is lacking.

CHAPTER THREE

Adoptive Transfer Of Autologous HIV-Specific CTL To A Patient With A Low CD4 Count

Introduction

As discussed in some detail in the introduction to this thesis, infection with HIV produces a state of cellular immunodeficiency in the affected patient eventually resulting in the development of opportunistic infections and tumours, which are the hallmarks of AIDS (Rosenberg and Fauci 1990). Therapy of HIV infection currently relies on better drug protocols for preventing opportunistic diseases and the use of combined anti-retroviral drug therapy, also known as highly active antiretroviral therapy (HAART). Recently, the use of HAART has been shown to decrease virus loads by 1-3 log-fold, increase survival (Mellors, et al. 1996) and has raised the possibility of eventual elimination of the virus (Perelson, et al. 1997). More recent data however, suggests that residual pools of virus latently residing in lymphocytes and macrophages remain relatively resistant to therapy. Whether these virus infected cells are accessible to CTL is not known.

Several independent observations suggest that CTL are critical for the control of HIV infection. In other viral infections of humans and animal models, CTL appear to protect from disease. For instance, protection from CMV has been shown to reside in Class I restricted cells (Quinnan, et al. 1980, Rook, et al. 1984) and the adoptive transfer of CD8+ cells results in clearance of murine CMV infection (Reddehase, et al. 1987). Similarly, protection from influenza appears to be mediated by Class I restricted CD8+ lymphocytes (Lin and Askonas 1981).

Virus-specific CTL are present early in HIV infection and are temporally associated with decreases in the plasma virus load following primary infection (Borrow, et al. 1994, Koup, et al. 1994).

In addition, these early and vigorous responses have also been shown to drive the selection of viruses with mutations in the targeted epitopes, thereby providing convincing evidence for CTL-directed pressure (Borrow, et al. 1996, Price, et al. 1997). During the asymptomatic phase of infection, HIV-specific CTL are present at high frequencies (Langlade-Demoyen, et al. 1988, Nixon, et al. 1988, Riviere, et al. 1989, Walker, et al. 1987) and a decline in CTL activity is associated with transition to AIDS and increasing morbidity (Klein, et al. 1995). It has also been shown that patients with higher frequencies of Env-specific memory CTL had a statistically significant median level of plasma HIV RNA about 1/3 that of patients with lower frequencies (Musey, et al. 1997). CTL responses have also been detected in infants and sex workers who had been exposed to HIV but who remained free of the virus, suggesting that CTL may play a role in preventing infection in some patients (Rowland-Jones, et al. 1993, Rowland-Jones, et al. 1995) and in a closely related animal model, the inhibition of CTL by the infusion of CD8-blocking antibodies was shown to result in marked elevations of simian immunodeficiency virus (SIV) in the plasma and lymph nodes of infected macaques (Matano, et al. 1998). More recently, a strong inverse correlation between numbers of circulating HIV-specific CTL and plasma viral load has been demonstrated (Ogg, et al. 1998). Taken together, these and other reports are consistent with the view that CTL responses are an integral part of virus control (Rowland-Jones, et al. 1997).

Adoptive cell transfer has been well-documented in animal models (reviewed in Greenberg 1991, Riddell and Greenberg 1995). Cell transfers in cancer patients have consisted of two effector populations. The first are lymphokine activated killer (LAK) cells which are generated by short term culture of peripheral blood lymphocytes in the presence of high concentrations of IL-2. These cells lyse transformed target cells and have minimal activity against normal tissues (Grimm, et al. 1982, Sondel, et al. 1986). Up to 10^{11} LAK cells, generated in vitro, have been given to patients in a single intravenous dose. Patients experienced only minor

symptoms and no pulmonary compromise demonstrating the safety of these infusions (Rosenberg, et al. 1987, Rosenberg, et al. 1985)

The second population are *in vitro* expanded lymphocytes derived from tumours. Such tumour-infiltrating lymphocytes (TIL) have been shown, in a murine model of adoptive transfer, to be 50-100 times more effective than LAK cells in eradicating micrometastases (Rosenberg, et al. 1986). TIL lines can be expanded to 10^{11} cells over a period of 3-8 weeks and some lines possess lytic activity which is restricted to autologous but not allogeneic tumour target cells while others, like LAK, lyse autologous and allogeneic cells equally well (Itoh, et al. 1986, Kradin, et al. 1987, Topalian, et al. 1987). Adoptive transfer of 5×10^{10} TIL cells along with IL-2 has resulted in toxicities which were attributable to IL-2 alone (Rosenberg, et al. 1988, Topalian, et al. 1988). Indium-111 labelling of TILs showed initial localization in the lungs, liver and spleen two hours after infusion and localization at sites of metastatic tumour by 24 hours (Fisher, et al. 1989).

And as described in the introduction, adoptive cell transfer has been successfully attempted using CMV and EBV-specific CTL. However, in contrast to adoptive cell transfer studies in patients at risk of CMV or with EBV infection, there are a number of considerations unique to adoptive cell transfer for HIV infection. Because many, if not most of the host cell targets of HIV are part of the immune system, CTL recognition and destruction of infected cells may result in destruction of macrophages and also CD4+ cells. This raises the possibility that massive CTL targeting of infected CD4+ cells may drastically reduce the CD4+ population. Nonetheless, the underlying assumption is that these CD4+ cells are infected, dysfunctional and productive of virus. Thus, destruction could only be beneficial.

Destruction of CD4 cells also results in the release of infectious virions, though possibly less than if viral-mediated lysis were able to progress naturally. The half-life of infected target cells subject to CTL-mediated lysis *in vitro* (at effector:target ratios

which are present *in vivo*) has been calculated to be around 0.7 days and thus in theory, HIV-specific CTL are present in sufficient numbers and act with sufficient kinetics to contribute to the lysis of infected cells and a decrease in the production of infectious virus (Klenerman, et al). Using these calculations, if the CTL to target ratio could be raised several-fold by adoptive transfer of HIV-specific clones, reduction of virus may be significantly affected, aside from the effects of disrupting infected cells prior to virus maturation.

Another consideration is that the survival and effector function of CTL may be highly dependent on CD4 cells - the very cells which are diminished in late HIV infections and AIDS. For instance, in mice with a null mutation of the CD4 gene, anti-viral CTL activity was diminished (Battegay, et al. 1994). Thus, in the setting of a low CD4+ count, adoptively transferred CTL may lack both optimal effector function and the ability to survive and expand efficiently.

Transfer of large numbers of specific CTL may also drive escape mutation of the virus. This was most clearly demonstrated by Koenig et al when they transferred 10^{10} CTL specific for an A3-restricted nef epitope (Koenig, et al. 1995). Prior to transfer, sequencing of the patient's virus revealed wild-type nef sequences; however, following two courses of nef-specific cell infusions, the majority of the virus population in the patient had mutations in and around the nef epitope.

We have examined the safety, feasibility and utility of CTL transfer to two HIV-infected patients. The data for the first patient (008) are presented in this chapter and the data for second patient (868) are presented in the next chapter. These two patients differ in a number of respects as outlined in Table 7. Perhaps most importantly, Patient 008 has a low virus load and (paradoxically) a low CD4 count while Patient 868 had a relatively high virus load and a high CD4 count. The importance of these observations will be discussed later.

Table 7: Clinical characteristics of adoptive transfer patients 008 and 868

| | Patient 008 | Patient 868 |
|--|-------------|-------------|
| Age | 34 | 34 |
| Years infected | 14 | 7 |
| CD4 cells/ μ l at time of infusion | 20 | 600 |
| RNA copies/ml at time of infusion | 30000 | 60000 |
| Antiretroviral therapy | None | AZT, DDI |
| Opportunistic diseases | None | HSV, PCP |

Two patients with AIDS, 008 and 868, were adoptively transferred with HIV-specific CTL over a period of two years. The patients differed with respect to CD4 cell count, viral load and anti-retroviral medications. herpes simplex virus (HSV), azidothymidine (AZT), didanosine (DDI), pneumocystis carinii (PCP).

Methods

Obtaining peripheral blood mononuclear cells

Peripheral venous blood was obtained through a #21 gauge butterfly needle into a 50 ml syringe. The blood was placed into sterile 50 ml conical tubes containing 50 µl of preservative-free heparin sulfate (Leo Laboratories, 1000 units/ml). Blood was diluted 1:1 with sterile RPMI and gently layered onto a density gradient (Lymphoprep) at a volume ratio of 2:1 blood to ficoll in either 25 ml universal tubes or 50 ml conical tubes. The tubes were centrifuged at 400 x g for 30 minutes at room temperature with the brake off. The buffy coat interface containing mononuclear cells was aspirated with a sterile transfer pipette, washed with sterile RPMI and centrifuged for 10 minutes at 800 x g. The cell pellet resuspended and washed again with RPMI and centrifuged a further 10 minutes at 400 x g. The cells were resuspended in RPMI containing either 10% fetal calf serum or 10% human serum, penicillin and streptomycin and glutamine (referred to as R10 or HSR10). The average yield from a healthy volunteer was 1 million PBMC per 1 ml of whole blood. Concentrated buffy coats obtained by leukapheresis were occasionally bought from the Oxford Transfusion Service. These yielded 3-5 million PBMC per ml of buffy coat.

Establishing B-cell lines (lymphoblastoid cell lines, LCL)

A minimum of two and a maximum of ten million PBMC were pelleted in a universal tube and resuspended in a 1 ml aliquot of supernatant obtained from an EBV-producing marmoset cell-line B958 and incubated for 1 hour at 37 °C in an atmosphere of 5% CO₂. The PBMC were then resuspended in 9 mls of RPMI containing 15% fetal calf serum, penicillin and streptomycin and glutamine and 1 µg/ml cyclosporin A (for suppression of T-cell growth). The suspension was placed in a 50 ml tissue culture flask and incubated at 37 °C in an atmosphere of 5% CO₂. B-cell lines took from 2 weeks to 6 weeks to become fully established. R10 medium was added to the cells periodically. When B-cell lines were established, they were grown in 200 ml flasks in a volume of approximately 20 mls.

Cell irradiation

In order to prevent ongoing mitosis and expansion of feeder cells, both PBMC and BCL were irradiated with a total of 3000-5000 rads in a Cesium gamma-irradiator (Atomic Energy Commission of Canada).

HLA typing

HLA typing was done using an amplification refractory mutation specific (ARMS) method developed by Bunce et al (Bunce, et al. 1995). Briefly, genomic DNA was extracted using a Puregene DNA kit (Gentra, Madison, Wisconsin). Genomic DNA was used as a template in the polymerase chain reaction (PCR) amplification of the HLA region (exons and introns) using allele-specific primers and controls. Amplification products were separated by agarose gel electrophoresis and photographed under ultraviolet light. Lanes containing positive bands were noted and interpreted according to a key in order to determine the HLA Class I haplotype. Separate HLA Class I subtyping reactions were performed as required.

Production of CTL lines

CTL lines were either produced through bulk culture in which autologous HIV infected lymphocytes served to stimulate CTL or through peptide-specific stimulation. A pellet consisting of two to five million PBMC was incubated with 100 μ M peptide in a volume of 100 μ l for 2 hours at 37 °C. The pellet was then resuspended in R10 and aliquotted into a 24 well plate at 2 million cells per well. IL-2 (25 cetus units/ml) or lymphocult-T (final concentration 10%, Biotest AG, Dreieich, Germany) was added to the cells three days later.

Bulk cultures were produced by resuspending approximately 5 million PBMC in 5 mls of R10 in a 50 ml flask overnight at 37 °C. Approximately 1 million cells were resuspended in 2 mls of R10 and 10 μ l of phytohemagglutinin (PHA, Murex) in a single well of a 24 well plate (roughly 5 μ g/ml). This has the effect of activating T lymphocytes, thus stimulating virus production in the infected cells. After 16 hours,

the PHA stimulated cells were washed once in sterile RPMI and added to the flask containing unstimulated PBMC. An aliquot of PHA blasts was frozen for PCR and virus sequencing.

Production of Clones

CTL clones were derived from CTL lines by a method of limiting dilution. Cloning mix consisting (per 10 mls of R10) of 10 million irradiated PBMC (from three or more different donors), 1 million irradiated, autologous B-cell lines pulsed with 1 - 100 μ M peptide and PHA (1:1000 dilution) was added to CTL such that when plating into a 96 well flat-bottom plate at 100 μ l/well the average number of CTL per well was 0.3, 3 or 30. Usually, two plates at each concentration were made. Lymphocult-T was added to each well at day 3 after stimulation (final concentration 10%) and the plates were left until day 14 at which time they were examined under an inverted microscope for evidence of T-cell growth. Positive wells were marked and expanded into 24 well plates (with cloning mix) a further 10 days and tested by cytotoxicity assay. In general, cells which grew rapidly did not lyse target cells as well as cells which grew slowly. Thus, the cloning plates were subjected to a second screen 20 days after stimulation and positive wells were again marked, expanded and tested. In addition, in accordance with a recent report (Alexander-Miller, et al. 1996), cloning with the use of lower concentrations of peptide (1 μ M or less compared to 100 μ M) often yielded clones which more efficiently lysed target cells.

Freezing and thawing cells

Two to twenty million cells were resuspended in 2 mls of freezing medium containing either 90% FCS (Sigma) and 10% dimethyl sulfoxide (DMSO) or 70% RPMI, 20% human serum and 10% DMS) and placed in a 2 ml freezing vial (Nunc). The vial was either placed on ice for 30 minutes or placed into a 4 °C isopropanol controlled rate freezing container, then placed in a -70 °C freezer overnight. After 16 hours the vials were transferred to liquid nitrogen tanks. Cells were thawed rapidly by placing frozen cryovials into a 37°C incubator and

resuspending the cells in cold R10. The cells were washed twice in R10 and plated at 2×10^6 cells/well of a 24 well plate with IL-2.

Peptide sythesis and HPLC

Peptides were synthesized by Karl McIntyre in the Institute of Molecular Medicine or purchased from Research Genetics (Alabama). In-house synthesized peptides were freeze dried and assayed for purity by high performance liquid chromatography (HPLC). Stock peptides were dissolved in DMSO to a concentration of 1 mg/ml and diluted into sterile RPMI for subsequent use.

CTL cytotoxicity assay

Effector cells were washed once, resuspended in R10, counted and made to a concentration of 1 million cells per ml. One hundred thousand cells (100 μ l) of this was placed into a U-well of a 96 well plate. Target cells were usually autologous transformed B-cell lines. B-cells were pelleted by centrifugation and resuspended in approximately 300 μ Ci of Cr-51 for 45 minutes at 37 °C. The cells were then washed twice with RPMI, the pellet was resuspended in 100 μ l of peptide at the relevant concentration and incubated at 37 °C for 1 - 2 hours. The cells were washed twice in RPMI and then resuspended in 1 ml of R10 and counted in a hemacytometer. The cells were made to a concentration of 100,000 per ml and placed into a U-well at a concentration of 5000 cells per well. Minimum lysis consisted of wells in which the target cells were in medium alone. Maximum lysis consisted of wells in which the target cells were lysed with 100 μ l of 5% triton-X. Target and effector cells were incubated together for 4-5 hours at 37 °C and 20 μ l of supernatant was harvested from each well onto a fibre mat. The mat was dried in a microwave oven and placed in a plastic bag with 10 mls of scintillation fluid and counted on a beta-plate counter. Lysis was calculated according to the formula:

$$\frac{\text{mean cpm target/effector well} - \text{mean cpm minimum lysis well}}{\text{mean cpm maximum lysis well} - \text{mean cpm minimum lysis well}} \times 100 (\%)$$

Preparing human serum

Human serum was prepared from volunteers who fasted 16 hours prior to phlebotomy as lipids appear to interfere with optimal CTL growth (K. Watanabe, Fred Hutchison Research Center, personal communication). Approximately 200 mls of blood was drawn serially into 50 ml syringes and placed in sterile 50 ml conical tubes containing no heparin. The tubes were left at room temperature for six to eight hours and then centrifuged at 2500 RPM in a Beckman centrifuge for 20 minutes at room temperature. The serum was drawn through a sterile plastic straw (Kwill) into a 50 ml syringe and pooled into a sterile 50 ml tube. The tubes were centrifuged for a further 10 minutes and the serum was decanted from the red blood cell pellet and placed in 65 ml aliquots. One ml aliquots of serum were saved for HIV, HBV and HCV serology. The 65 ml aliquots were heat inactivated in a 58 °C waterbath for 1 hour and frozen at -20 °C.

Large scale expansion of CTL for infusion

Large scale expansion of CTL was based on the methods of Riddell and Greenberg (Riddell, et al. 1992, Walter, et al. 1995). Briefly, CTL were grown upright in T25 or T75 tissue culture flasks. Each flask contained the T-cell clone, irradiated mixed allogeneic PBMC and mixed allogeneic irradiated LCL which acted as feeder cells. TCR stimulation was provided by monoclonal anti-CD3 (OKT3) and HSR10 was the culture medium. The details of additions are in Table 8 and a more extensive discussion of CTL expansion follows.

Large scale expansion of CTL clones or oligoclonal lines is technically straightforward but not always successful. To a large extent, the nature of the clone, which is to be expanded, is important. We and other investigators have found that clones which have received repeated TCR stimulation do not expand with the efficiency of recently cloned cells (Dr. S. Riddell, personal communication). Thus, ideally, the CTL should undergo one large scale expansion following their initial cloning, and aliquots of these cells may then be cryopreserved for future expansions.

Table 8: *In vitro* CTL expansion protocol

| | T25 flask | T75 flask |
|----------------------------|-----------------------------------|-----------------------------------|
| T-cell clone | 5×10^4 - 1×10^5 | 2×10^5 - 5×10^5 |
| Irradiated allogeneic PBMC | 25×10^6 | 75×10^6 |
| Irradiated allogeneic LCL | 5×10^6 | 15×10^6 |
| Anti-CD3 (OKT3) | 30 ng/ml | 30 ng/ml |
| RPMI with 10% Human Serum | | |
| Total Volume | 25-30 mls | 50-60 mls |

CTL were expanded in number *in vitro* prior to adoptive transfer. CTL clones were cultured in either T25 or T75 flasks for a period of 14 days using irradiated lymphoblastoid cell lines (LCL) and peripheral blood mononuclear cells (PBMC) as antigen presenting and feeder cells, in medium supplemented with human serum and IL-2. Further details are described in the text.

In addition, CTL which are derived from HIV-infected patients whose CD4 cell counts are below 200/ μ l do not appear to grow as vigorously. In general, cells grow more successfully in smaller flasks (eg. T25 (50 ml) flasks vs. T200 (600 ml) flasks). However, the use of the T25 flasks requires the manipulation of numerous flasks increasing both the labour involved and the chance of bacterial or fungal contamination. Thus, in practice, the use of T75 (200 ml) flasks represents an acceptable balance between cell growth and manual labour. Large numbers of LCL and PBMC are required for large scale expansion and these may be prepared beforehand and frozen. PBMC are best generated by ficoll separation of leukapheresis-prepared buffy coats which may be obtained from the local blood authority.

Equipment and reagents required for large scale expansion

- CTL clone (2 million cells)
- anti-CD3 (OKT3, Orthoclone) stock 1 μ g/ml
- irradiated, syngeneic or allogeneic LCL (150 million cells)
- irradiated, allogeneic PBMC (750 million cells)
- 1 x T200 tissue culture flask
- 10 x T75 tissue culture flasks (Costar)
- Plastic Universal tubes (25 mls) or conical tubes (50 mls)
- hemocytometer
- RPMI 1640 medium supplemented with glutamine, penicillin, streptomycin and 10% human or fetal calf serum

Preparation of the cells and reagents

- i) In a T200 sterile flask, combine CTL clone, PBMC and LCL in 100 mls of R10.
 - ii) Add 15 μ g of anti-CD3 (final concentration = 30 ng/ml) and bring volume up to 500 mls with R10.
3. Distribute cell mixture to 10 x T75 flasks, 50 mls per flask.

4. Incubate flasks upright at 37 °C, 5% CO₂ overnight.
 - i) Add IL-2 (human recombinant 25 units/ml) to flasks the next day.
 - ii) Five days after initial setup, harvest cells from all flasks, wash once with RPMI and resuspend in R10/IL-2 at 50 mls per flask x 10 flasks.
 - iii) Eight days after setup, resuspend the cells in a representative flask and count. If the cells concentration is $> 2 \times 10^6$ per ml, split 1 flask to two flasks.
8. Test cells for CTL activity at 12-14 days.

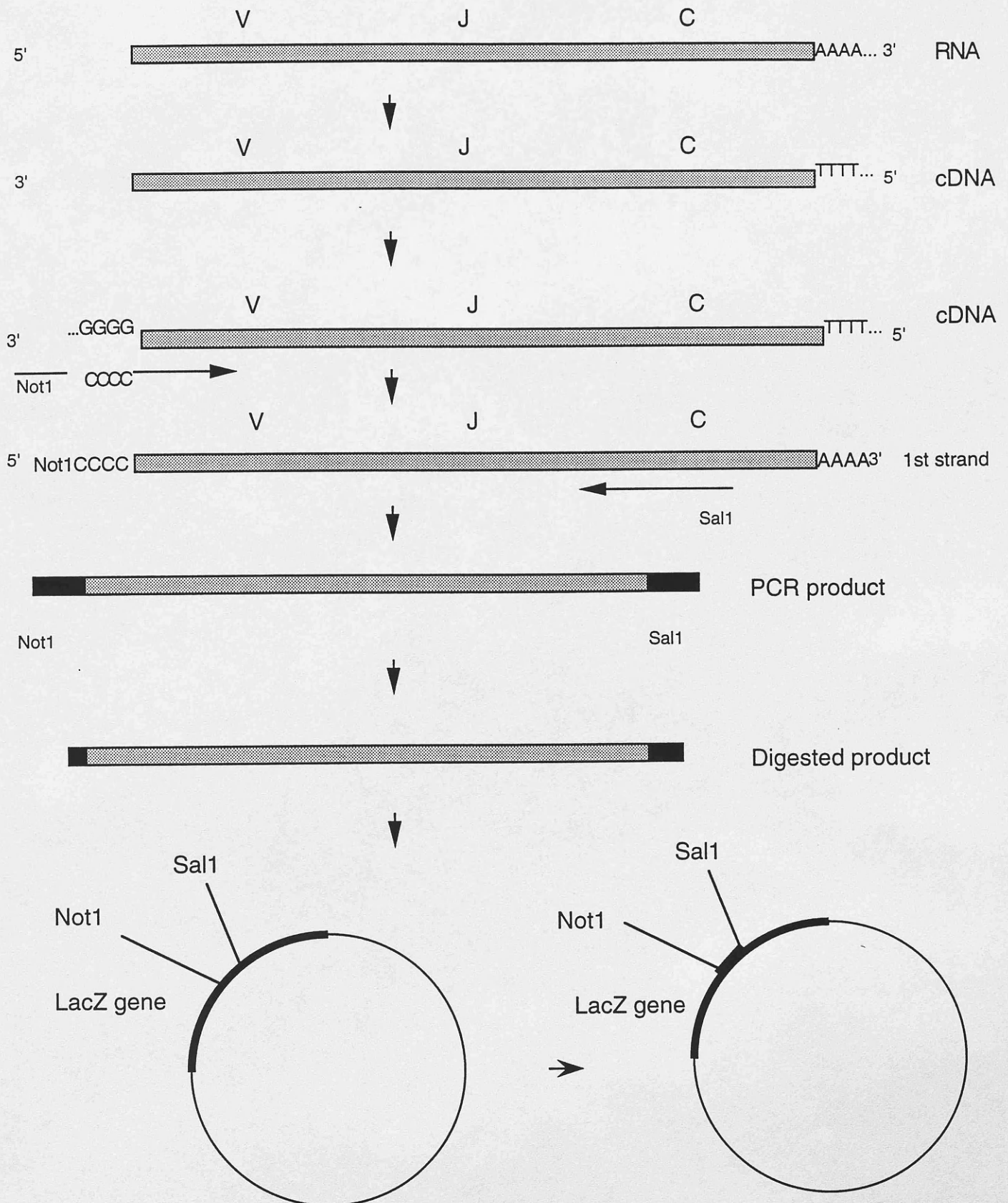
Anchored PCR and TCR cloning

The anchored PCR method has been previously described (Loh, et al. 1989, Moss, et al. 1995). An overview is presented in Figure 7.

Figure 7: Anchored PCR method overview

CTL clonal cDNA was produced from oligo-dT primed RNA. A poly-G tail was added with terminal transferase and PCR to amplify the CDR3 region was primed by oligonucleotides complementary to the constant regions of V-beta and V-alpha and the poly-G tail. The amplified product was purified from the gel and cloned into phagescript vector and the nucleotide sequence was determined by dideoxynucleotide sequencing.

Figure 7: Anchored PCR Method



RNA was prepared from 1-5 million CTL using RNazol or TRI (Sigma). Oligo dT (Collaborative Research) primed cDNA was synthesized with MMLV reverse transcriptase. The cDNA product was ethanol precipitated three times and then a poly G tail was added to the 5' end using terminal transferase. The poly G cDNA was extracted with phenol/chloroform and used in a template in a PCR reaction. The poly G tail serves as an anchor a poly C primer and a constant alpha or beta region primer. Primers were as follows:

Poly-C anchor 5' gca ttc agc tgc ggc cgc (c)₁₄ 3' (includes Not1 site)

A Sal AMP (a) 5' tga ccg cag tcg aca gac ttg tca ctg gat t 3'

Cb AMP Sal (b) 5' ata ctg gag tcg acg gag atc tct gct tct gat g 3'

The poly-C anchor contains a 5' Not 1 site and the Calpha and Cbeta anchors each contain a 5' Sal site. The cycling parameters were:

1 cycle 94° for 4 minutes

5 cycles 94° for 1 minute
65° for 1 minute
72° for 2 minutes

35 cycles 94° for 1 minute
58° for 1 minute
72° for 2 minutes

PCR products were purified through a Wizard Prep mini-column (Promega) and a small aliquot was run on a 1.5% agarose/TBE gel. A 600 base pair product corresponded to the TCR PCR product.

The PCR product was then digested at 37 °C overnight with Not 1 and Sal. The digested product was separated on a 6% acrylamide gel and the relevant band was

identified by ethidium bromide staining, excised and eluted overnight in acrylamide elution buffer. The DNA was ethanol precipitated and a ligation reaction consisting of insert DNA, Phagescript SK vector and T4 ligase (Boehringer) was performed. Ligated vector was transformed into competent XL1-MRF' E. coli with blue-white selection. White colonies containing insert were grown in XL1 bacteria until OD600=0.5 and single-stranded phage DNA was precipitated from the supernatants. Single-stranded DNA was used as a template in dideoxy nucleotide sequencing (USB Sequenase 2.0).

Flow cytometry

Cells were pelleted in a universal tube (800 x g for 5 minutes) and resuspended in PBS/BSA at a concentration of 3-5 million/ml. Aliquots of 100 µl were placed in the round bottom wells of 96 well plates according to the number of staining conditions. The plate(s) was spun in a centrifuge for 5 minutes, the supernatant "flicked" out and the plate subjected to vortexing. The cells were then suspended in PBS/1%BSA/1% human serum/1% mouse serum and stained with the relevant antibodies for 30 minutes on ice. For double or triple staining the cells were washed twice and pelleted between labellings. Cells were resuspended in 400 µl of PBS/1% FCS/1% formaldehyde and analyzed on a Becton-Dickinson FACScan using Cellquest software.

Sterility Testing

Briefly, one ml of a given cell culture was placed in a sterile tube and centrifuged at 1000 x g for 10 minutes. The supernatant was decanted to a separate sterile tube. Both tubes were submitted to the microbiology lab for bacterial and fungal culture using a blood culture protocol. Aliquots of cells were also tested for mycoplasma and pelleted cells were submitted to virology for PCR testing of HBV, HBV and EBV. A more detailed discussion of safety issues follows.

The two paramount considerations in transferring cultured cells to patients are the sterility of the preparation and the absence of transformed cells. Because the

population receiving adoptive cell therapy is often immunosuppressed, any transferred organisms may produce serious infections. The cells and growth medium must be periodically assayed for bacterial, viral and fungal contamination. However, because CTL may be infected at anytime up to the moment of transfer and because the culture of fastidious organisms may require days to weeks of incubation, it is usually necessary to infuse cells prior to complete culture results. Hence, meticulous handling of cells is an absolute requirement. One way of avoiding this problem is to cryopreserve cells at their peak of lytic activity and thaw them just prior to transfer, when the microbiological results have been finalized. However, there is often a reduction in cytotoxic activity as well as a loss of absolute cell numbers in lymphocytes which have been frozen. Organisms likely to contaminate cell cultures include mycoplasmas, yeast and other fungi. In our practice, we screen cells for contamination three days before and on the day of infusion by testing aliquots of cells for mycoplasma and sending samples to the clinical microbiology laboratory for cultivation using a blood culture protocol. While not ensuring that samples are absolutely sterile at the time of infusion, the latter samples allow us retrospective diagnosis of any bloodborne infection. In addition, as mentioned in above, it is necessary to screen the human serum used in cell culture media for bloodborne viruses such as HIV, HBV and HCV.

Most protocols for lymphocyte expansion utilize EBV transformed B cells (LCLs) as either antigen presenting cells and/or a source of costimulatory molecules. These cells must be thoroughly irradiated prior to use to prevent the escape of any transformed cell (4000-8000 rads). One method of testing whether the B cells have been sufficiently irradiated is to maintain an aliquot in appropriate medium and observe the cells for outgrowth. Other investigators have also taken the additional precaution of growing LCL in acyclovir-containing media thus preventing the formation of late lytic cycle infectious EBV particles (Haque, et al. 1998).

Virus loads

HIV RNA plasma titre was determined using the HIV Amplicor kit from Roche. Briefly, RT-PCR was performed on 200 µl of EDTA plasma and controls. PCR product was purified and applied to 96 well microtitre plates coated with complementary oligonucleotide-biotin complex. The plates were washed and product was detected with a streptavidin-HRP colour indicator. A standard curve of RNA copies per ml was constructed from the control RNA extractions and patient samples were plotted.

Patient Consent

Ethical approval was obtained from Central Oxford Regional Ethics Committee (COREC) and informed consent from the patient.

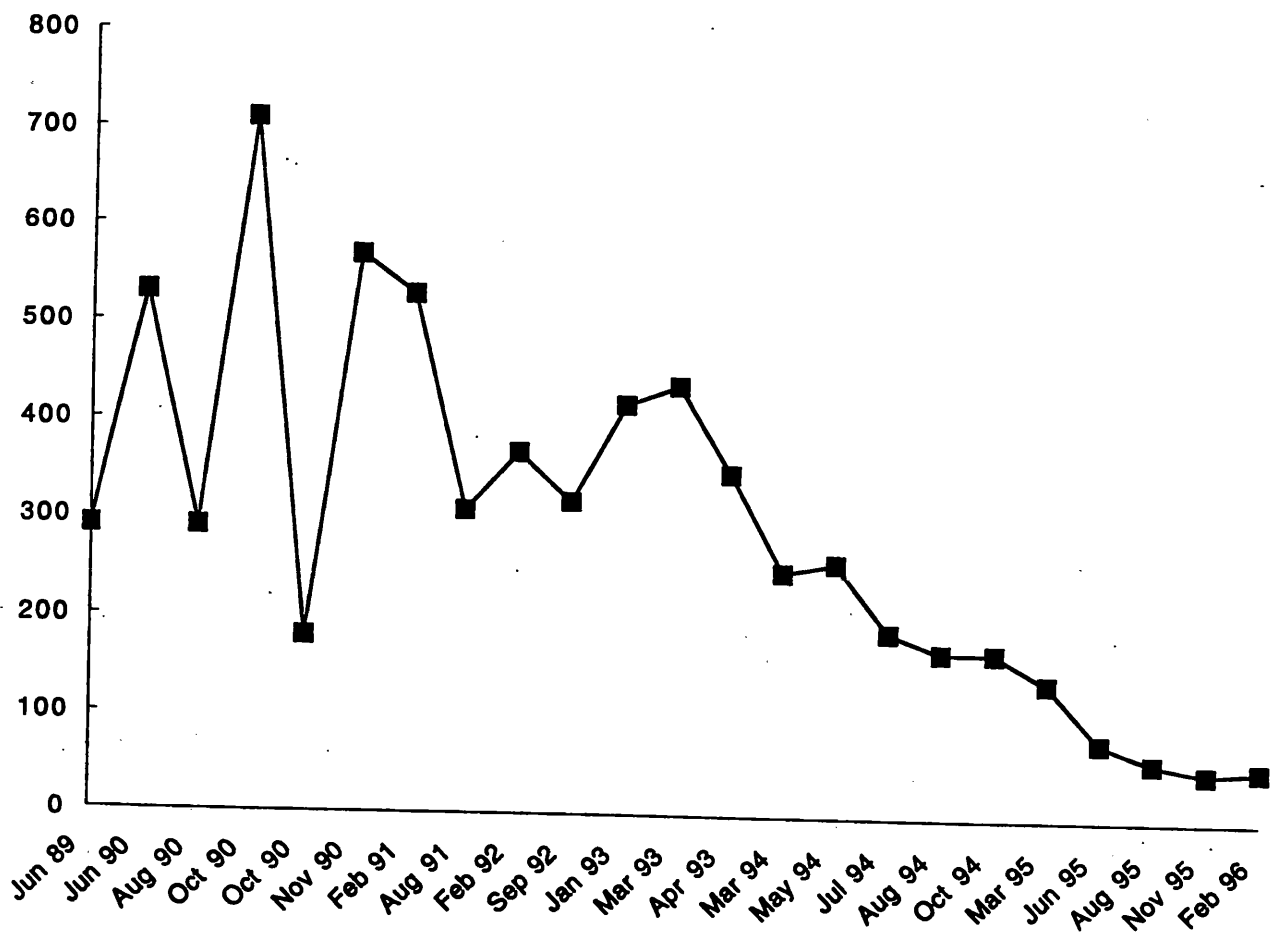
Results

Patient 008

Patient 008 was a 34 year old white male hemophiliac who contracted HIV from contaminated Factor VIII concentrate infusions approximately 14 years prior to this study. At the time that we first began studying him, his CD4 count was > 500/µl and he was receiving no medications. At the time when this adoptive transfer study was being planned, his CD4 count had declined to approximately 100/µl and at the actual time of infusion, the CD4 count was 20/µl. At no time during the infusions did he develop any opportunistic infections or receive any anti-retroviral medication. His declining CD4 lymphocyte count is shown in Figure 8.

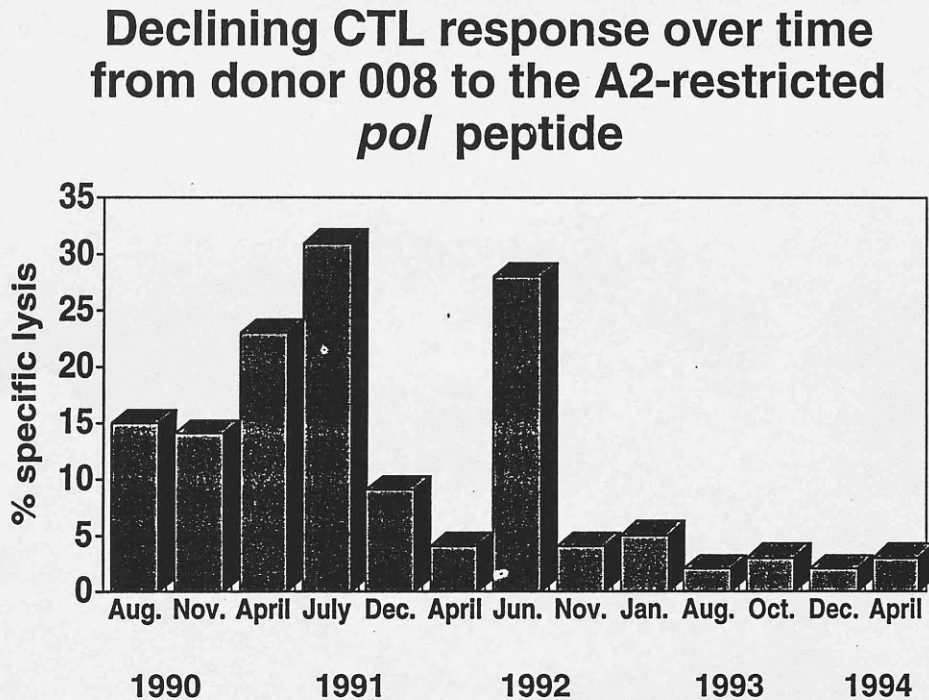
Importantly, Patient 008 seemed to have lost over time his CTL responses to Pol and to Gag (Figure 9 and personal communication, Sarah Rowland-Jones). Thus, there appeared to be an opportunity to restore his CTL responses by adoptive transfer of CTL.

Figure 8: Patient 008 CD4 count



CD4 cell counts from Patient 008 for an 8 year period preceding adoptive transfer of CTL. Counts are expressed as cells/ μ l.

Figure 9: Patient 008 Historical CTL Responses to Gag & Pol



PBMC from Patient 008 were restimulated with Pol peptide (ILKEPVHGV) at the indicated timepoints and tested for CTL activity for a 4 year period preceding adoptive transfer.

HIV-specific CTL clones derived from Patient 008

Three CTL clones, derived from Patient 008 in 1991 by Stephen McAdam, were thawed, cultured and tested for CTL activity (Table 9, Clones 18,20,28). Clone 18 did not grow well initially and the other two clones were considered for expansion and infusion. Clone 28 which recognizes an HLA B8-restricted nonamer derived from Gag p24-13 (GEIYKRWII) was an excellent killer cell line with 60-80% specific lysis at a 2:1 effector-target ratio (Figure 10). In contrast, Clone 20 which recognizes an A201-restricted nonamer derived from the pol product of HIV (ILKEPVHGV) had a lower level of specific lysis (Figure 10). Both clones also recognized HIV variants (GDIYKRWII and ILKEPVHEV respectively) which had previously been sequenced from the patient.

Table 9: HIV-specific CTL clones derived from Patient 008

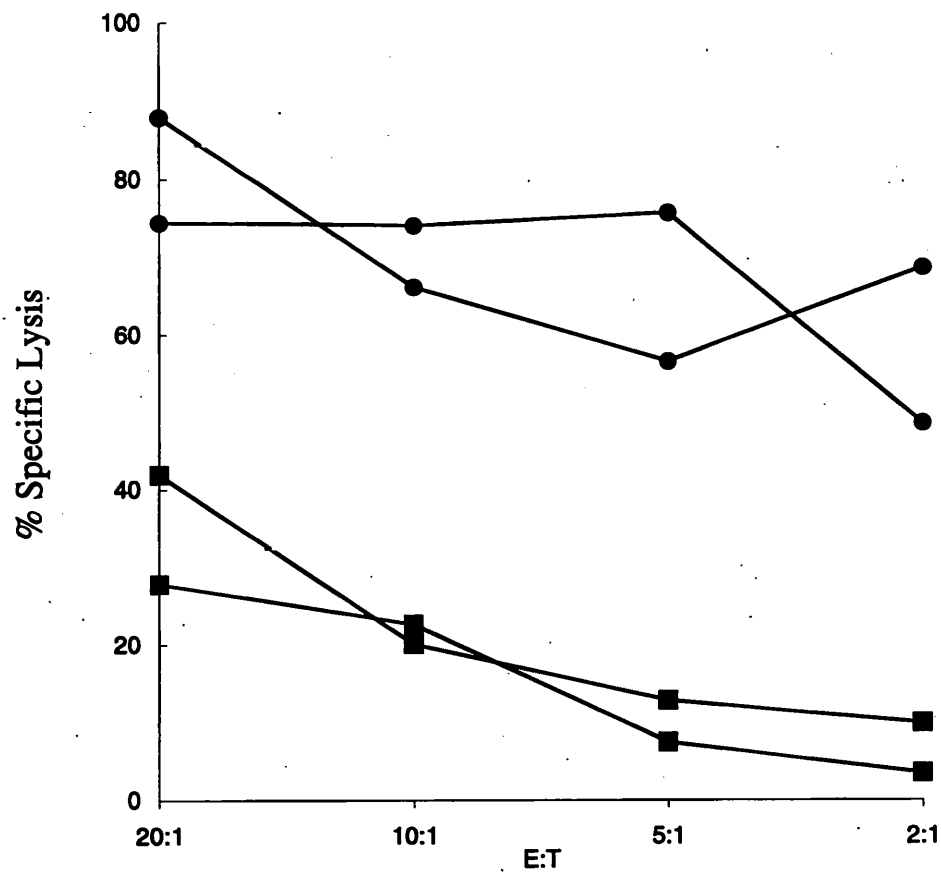
| Clone | HLA | Epitope | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|-------|-----|---------|---|-----|---|---|-----|---|---|-----|---|
| 18 | B8 | p17 | G | G | K | K | K/R | Y | K | L | |
| 20 | A2 | pol | I | L | K | E | P | V | H | G/E | V |
| 28 | B8 | p24 | G | E/D | I | Y | K | R | W | I | I |
| 41 | B8 | p24 | G | E/D | I | Y | K | R | W | I | I |

CTL clones previously derived from Patient 008 recognized the indicated epitopes from Pol, Gag p17 and Gag p24 restricted by HLA A201, B801 and B801 respectively. These clones were tested for CTL activity before cryopreservation. The original nomenclature for the clones is retained in the text. Clones also recognized the indicated variant peptides sequenced from Patient 008.

Figure 10: Activity of the HIV-specific CTL clones from Patient 008

The CTL clones described in Figure 9 (Clones 20 and 28) were thawed, recultured and tested in duplicate for CTL activity against autologous target LCL coated with 1 μ M of indicated peptide at different effector:target ratios. Clone 20 (HLA A2 Pol restricted) is represented by dark squares while Clone 28 (HLA B8 Gag restricted) is represented by dark circles.

Figure 10: HIV-specific CTL clones from Patient 008



Anchored PCR for V-alpha and V-beta clone sequences

Both clones were subjected to anchored PCR in order to:

1. confirm their clonality
2. determine their V-beta family
3. identify their specific VDJ/CDR3 sequences

The V-alpha and V-beta CDR3 sequences for both clones are shown in Figure 11. Clone 20 was determined to be Vb2.1/Jb1.2, Va17/Ja9.16 and Clone 28 was Vb16.1/Jb2.2, Va11/Ja13.2/Va7/Ja15.3. The two V-alpha sequences for clone 28 reflect the fact that although allelic exclusion restricts a lymphocyte to expressing only a single V-beta recombinant, two V-alpha chains both of which may pair with the V-beta chain, are permissible. At least 16 cDNA-transformed clones were sequenced for each CTL line and these were the only in-frame sequences obtained.

Flow cytometry of clones

The CTL clones were then subjected to flow cytometry in order to further characterize their phenotype. The primary FACS data are shown in Figures 12 and 13. Clone 28 was the expected phenotype, with surface expression of CD8 and V-beta 16 but not CD4 or V-beta 2. In contrast, Clone 20 was unexpectedly mixed, with 85% of the line expressing CD4 (and 15% expressing CD8) and 80% of the line expressing V-beta 2.1. This phenotype is consistent with a oligoclonal line consisting of both a CD4 and CD8 clone.

Magnetic bead separation of Clone 20

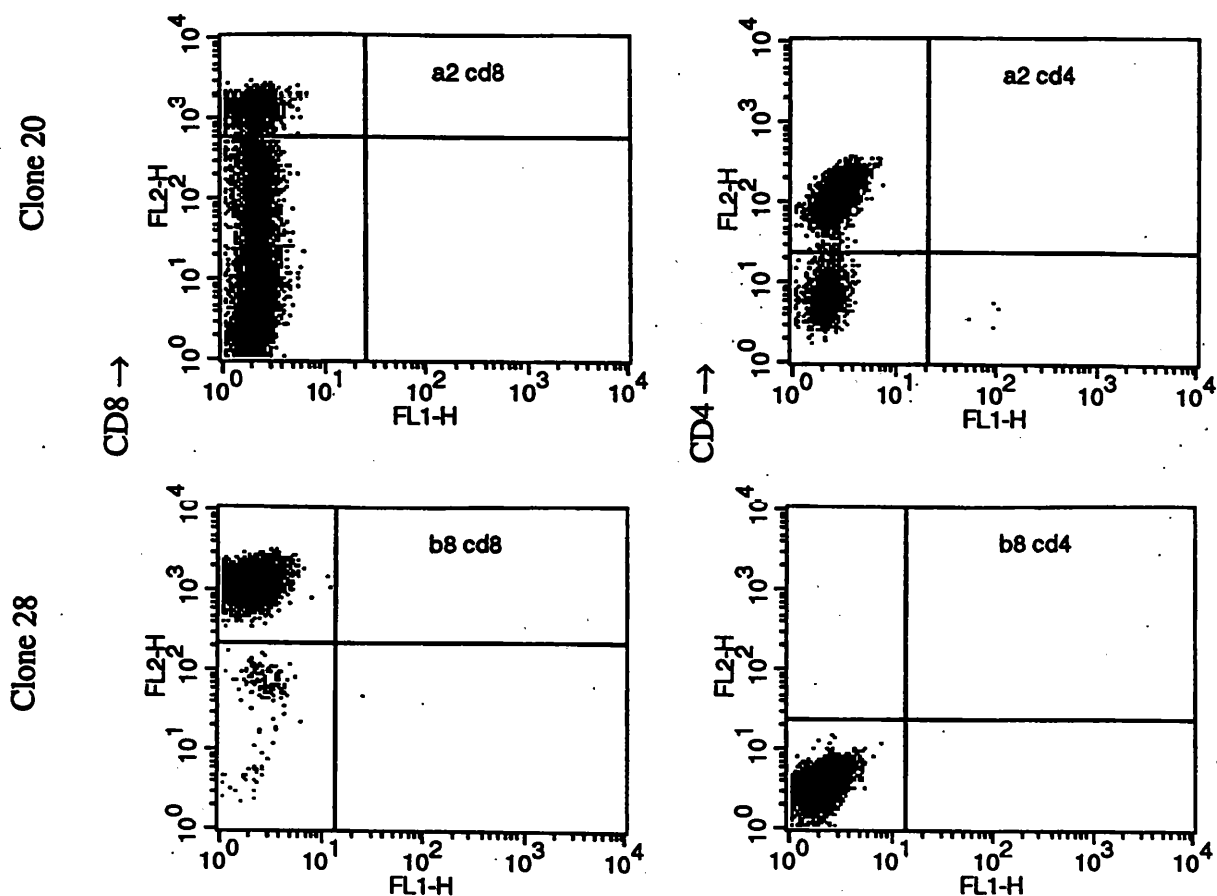
Clone 20 (HLA A2-pol specific) was separated into CD4 and CD8 fractions using monoclonal antibodies bound to magnetic beads. The separated fractions were then tested for CTL activity (Figure 14). Clone 20 (before fractionation) had a specific lysis of 25% while the CD4 and CD8 fractions respectively had specific lysis of 9% and 45%. Thus the CTL activity of Clone 20 resides in the CD4-CD8+ fraction of the cells. These data serve to explain some of the phenomena observed:

Figure 11: CDR3 sequences of infused clones

| Clone | Subset | Number | CDR3 Region |
|-------|--------------|--------|--------------------------------|
| 20 | Va17/Ja9.16 | 6/8 | --V-----D-----J----- |
| 28 | Va11/Ja13.2 | 6/8 | GDSATYFCAASIRGGSEKLVFGKGTKLTV |
| | Va7/Ja15.3 | 2/8 | ADAAVYYCAVEDGGNFVFGPGTRLVLPY |
| | | | KDSASYLCAVSAGGASGGSYIPTFGRGTS |
| 20 | Vb2.1/Jb1.2 | 14/16 | --V-----D-----J----- |
| 28 | Vb16.1/Jb2.2 | 14/14 | HPEDSSFYICSALEGGGYTFGSGTRLTV |
| | | | ELED SGVYFCASSYLLRTGDTRELFEGEG |

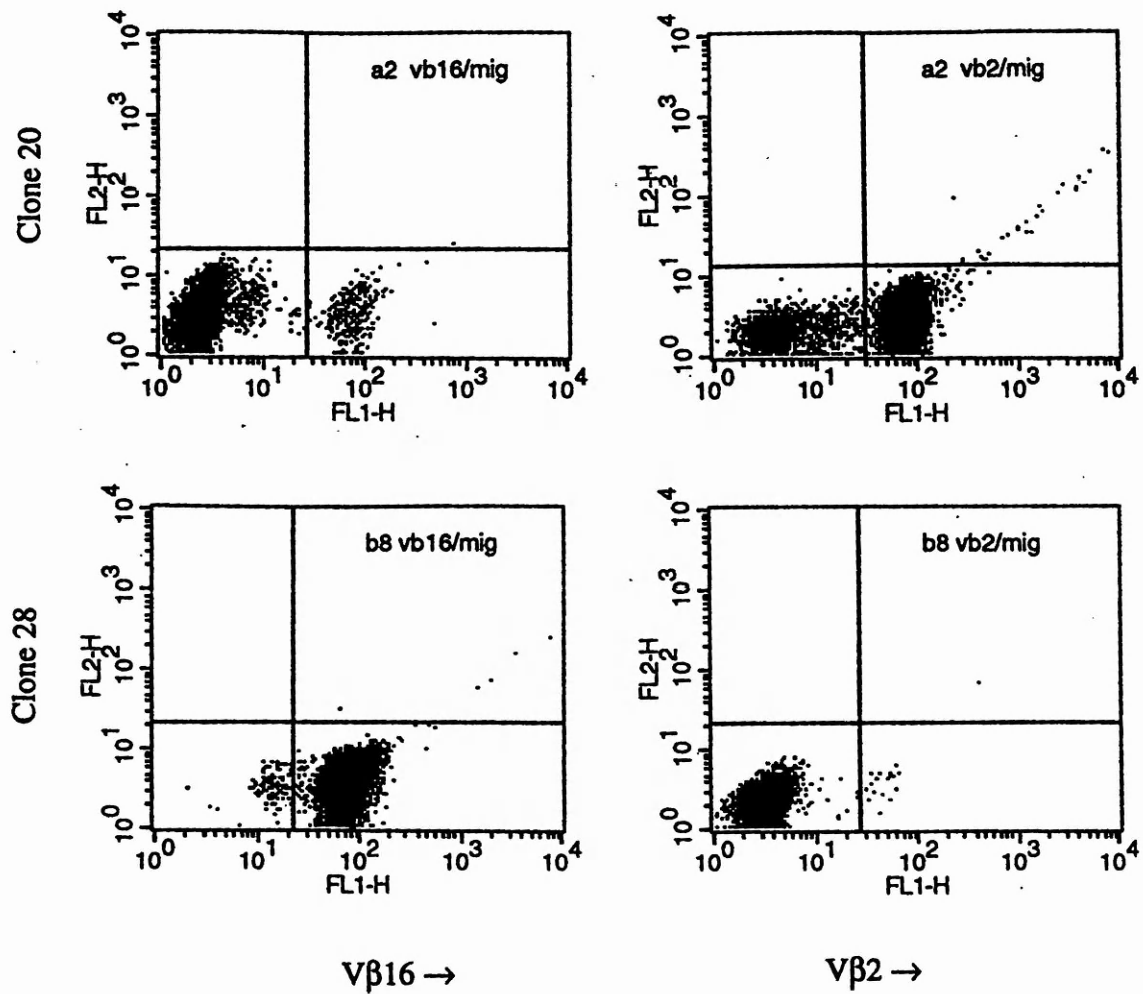
The nucleotide sequences of the V-alpha and V-beta complementarity-determining 3 (CDR3) segments (containing Variable, Joining or Diversity regions) of Clone 20 (A2, Pol) and Clone 28 (B8, Gag p24) were determined by anchored. Number refers to number of transformed colonies sequenced.

Figure 12: CD4/8 Phenotype of Patient 008 Clones 28 and 20



Immunophenotype of Clone 20 (A2 Pol), top two panels and Clone 28 (B8 Gag p24) bottom two panels. FL2 refers to staining with either CD4 or CD8. a2 refers to Clone 20 and b8 refers to Clone 28.

Figure 13: TCR Phenotype of Patient 008 Clones 28 and 20



Immunophenotype of Clone 20 (A2 Pol), top two panels and Clone 28 (B8 Gag p24) bottom two panels. FL1 refers to staining with either V-beta 16 or V-beta 2.1 followed by anti-mouse Ig FITC.

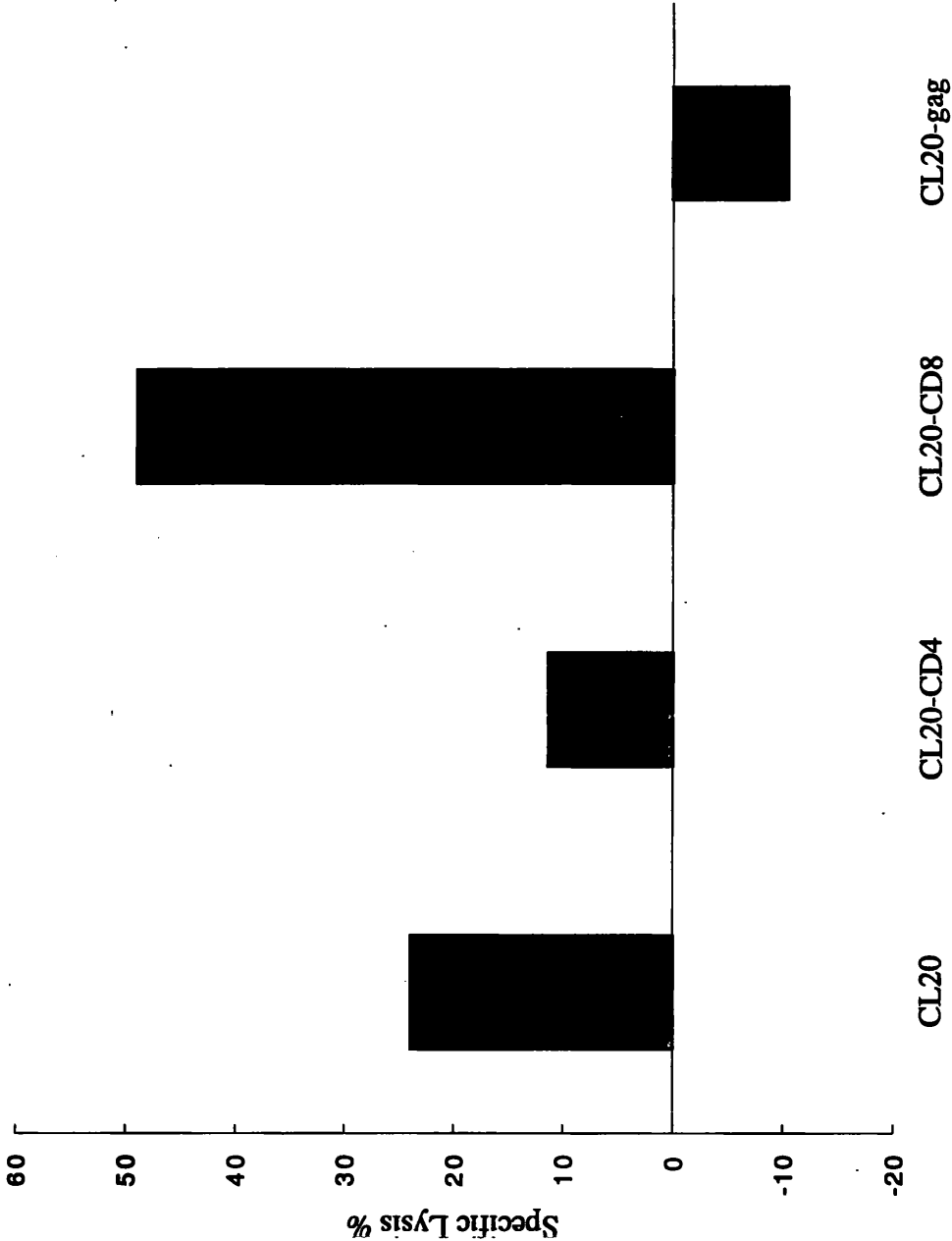
1. Clone 20 did not lyse cells as efficiently as normal clones.
2. Anchored PCR detected only a single V-beta CDR3 sequence.

Efforts to clone the CD8 expressing clone from this oligoclonal line yielded two separate clones, neither of which could be cultured efficiently.

Figure 14: CTL activity of CD4+ fraction of Clone 20

Clone 20 was fractionated using magnetic beads conjugated with antibodies to CD4 or CD8 and each fraction was tested for CTL activity against autologous LCL coated with 1 μ M of Pol peptide. CL20 is unfractionated Clone 20. CL20-CD4 refers to the CD4+ fraction of Clone 20 while CL20-CD4 refers to the CD4- fraction. CL20-gag refers to Clone 20 tested against autologous LCL coated with a control Gag peptide.

Figure 14: Patient 008 CTL Clone 20



First Infusion

Table 10 summarises the clones and cell numbers transferred into Patient 008. Clone 28 was expanded for the first infusion. Two weeks prior to infusion, the cells were cultured according to the protocol of Riddell et al. Aliquots of cells and supernatant were obtained on infusion day for microbiological and virological testing. Because of the theoretical risk of infusing irradiated autologous B cells, anti-EBV serology was obtained and the EBV IgG was positive. (Anti-EBV CTL lines could not be made from Patient 008 PBMC, Figure 15).

The cells were tested on Day 13 for CTL activity (Figure 16). Twenty-five million cells of Clone 28 were obtained for the first infusion. These were washed three times in PBS, resuspended in 250 ml of normal saline and transported to the ward for infusion. The cells were infused via a 21 gauge butterfly in the median vein of the antecubital fossa over a period of 15 minutes. Venous blood was obtained at various timepoints following infusion for cell counts, flow cytometry, virus loads and CTL assays.

Table 11 summarises the hematological and virological assays following the first infusion. The optimal timepoint for detecting any increases in leucocyte counts or in CD4+ or CD8+ cells would be shortly after infusion, but at one hour post-infusion, there was no significant rise in these cell numbers. Similarly, there were no significant decreases or increases in viral load followed up to one week post-infusion. In theory, the largest drop in virus load due to the cytotoxic activity of infused cells should occur mostly within the first 12 hours after infusion.

Lymphocytes obtained from the patient before and after infusion were also tested for cytotoxic activity against autologous transformed B lymphocyte cell lines exogenously loaded with relevant peptide. Patient lymphocytes were either tested immediately *ex vivo* (fresh CTL assay), an assay in which the PBMC are incubated directly with target cells or restimulated with peptide and IL-2 in an attempt to increase the precursor frequency of HIV-specific CTL, then assayed (cultured or

Figure 15: Patient 008 EBV CTL

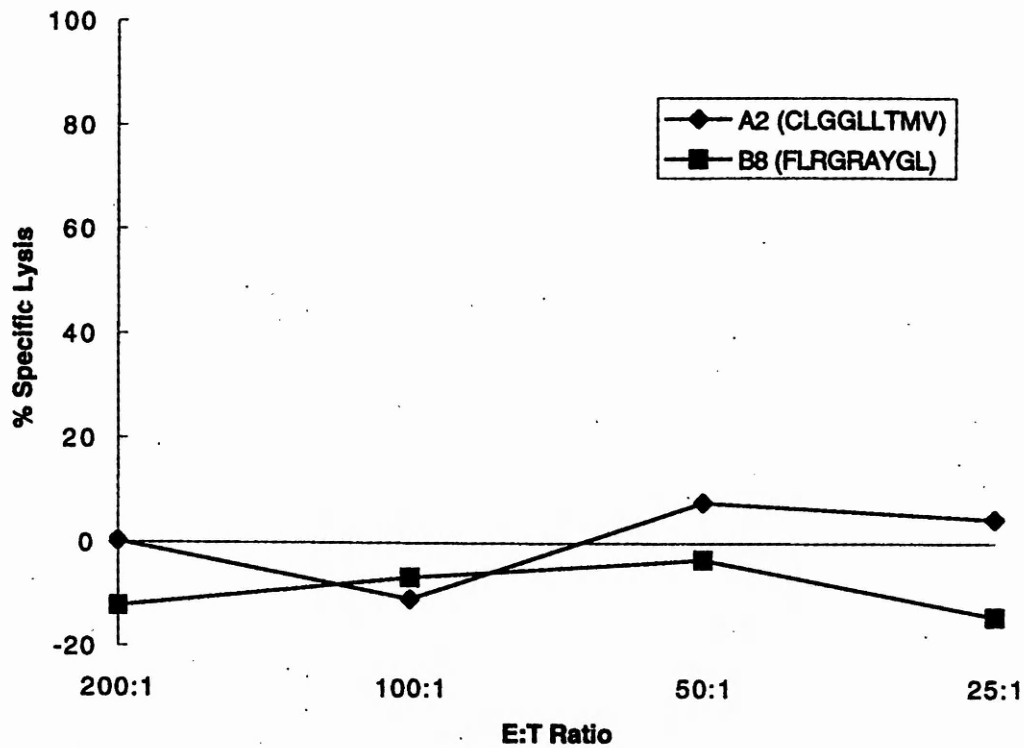


Figure 15 Patient 008 EBV-specific CTL activity

PBMC from Patient 008 were restimulated and cultured with the indicated peptide epitopes derived from Epstein-Barr virus (EBV) and tested for CTL activity against autologous LCL coated with the relevant peptide.

Figure 16: Patient 008 Clone 28

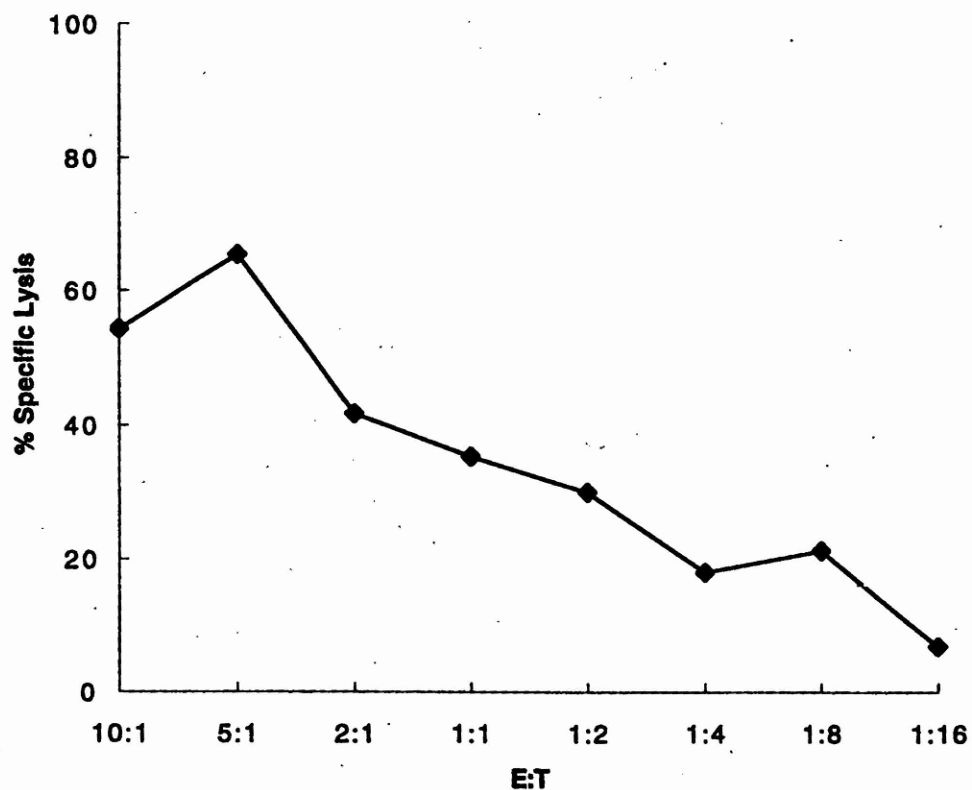


Figure 16: Patient 008 Clone 28 CTL activity pre-first infusion

The cytotoxicity of Clone 28 was tested on the day of infusion. Target cells were autologous LCL coated with 1 μ M of HLA-B8 restricted p24 peptide at the indicated effector:target ratios.

Table 11: Hematological and virological laboratory markers before and after the adoptive transfer HIV-specific CTL.

| a. First Infusion | 0 | +1hr | +4h | +20h | +7d |
|--------------------------|----------|-------------|------------|-------------|------------|
| WBC | 2000 | 1500 | | | |
| CD3 | 640 (68) | 600 (65) | | | |
| CD4 | 30 (4) | 20 (4) | | | |
| CD8 | 630 (63) | 600 (60) | | | |
| Virus (copies/ml) | 31,000 | 20,000 | 35,000 | 25,000 | 51,000 |

| b: Second Infusion | 0 | +1hr | +4h | +20h | +7d |
|---------------------------|----------|-------------|------------|-------------|------------|
| WBC | 1700 | 2000 | | | |
| CD3 | 410 (63) | 490 (68) | | | |
| CD4 | 30 | 20 | | | |
| CD8 | 380 | 450 | | | |
| Vb16 | 1.80% | 2.40% | | | |
| Vb17 | 5.40% | 5.40% | | | |
| Virus (copies/ml) | 56,000 | 49,500 | 49,000 | 66,000 | 59,000 |

| c. Third Infusion | 0 | +2hr | +6d | +18d |
|--------------------------|----------|-------------|------------|-------------|
| WBC | 1700 | | 2100 | |
| CD3 | 310 (64) | | 420 (67) | |
| CD4 | 10 (3) | | 20 (3) | |
| CD8 | 290 (60) | | 400 (63) | |
| Virus (copies/ml) | 49,000 | 41,000 | 65,000 | 84,000 |

Blood samples obtained from Patient 008 at each of the indicated timepoints were assayed for white blood cell (WBC) count and immunophenotyped for CD3, CD4, CD8 and in the second infusion, V-beta 16 and an irrelevant control, V-beta 17. Viral loads are expressed as RNA copies/ml. Percentages are expressed in brackets.

restimulated assays). Figure 17 shows the *ex vivo* activity of CTL before and after infusion. Figure 18 shows the restimulated CTL activity present in Patient 008 before and after cell transfer. Surprisingly, even *before* transfer, there was considerable activity (30% specific lysis) in response to target cells bearing the Gag p24 peptide.

Figure 17: Ex vivo “fresh” CTL activity post-first infusion

PBMC obtained from Patient 008 just before (Time 0) and at 1 hr and 20 hours after the adoptive transfer of CTL Clone 28 (HLA-B8 restricted, Gag p24-13) were tested immediately *ex vivo* for CTL activity against autologous LCL coated with the indicated peptides. Squares represent Clone 28 and diamonds represent Clone 20.

Figure 18: Restimulated CTL activity post-first infusion

PBMC obtained from Patient 008 before transfer (diamonds) and at 1 hr (squares), 4 hours (triangles) and 7 days (circles) after transfer were restimulated with autologous LCL coated with peptide Gag p24-13 and tested after 14 days of culture in the presence of IL-2.

Figure 17: Patient 008 First Infusion Ex Vivo CTL Assays

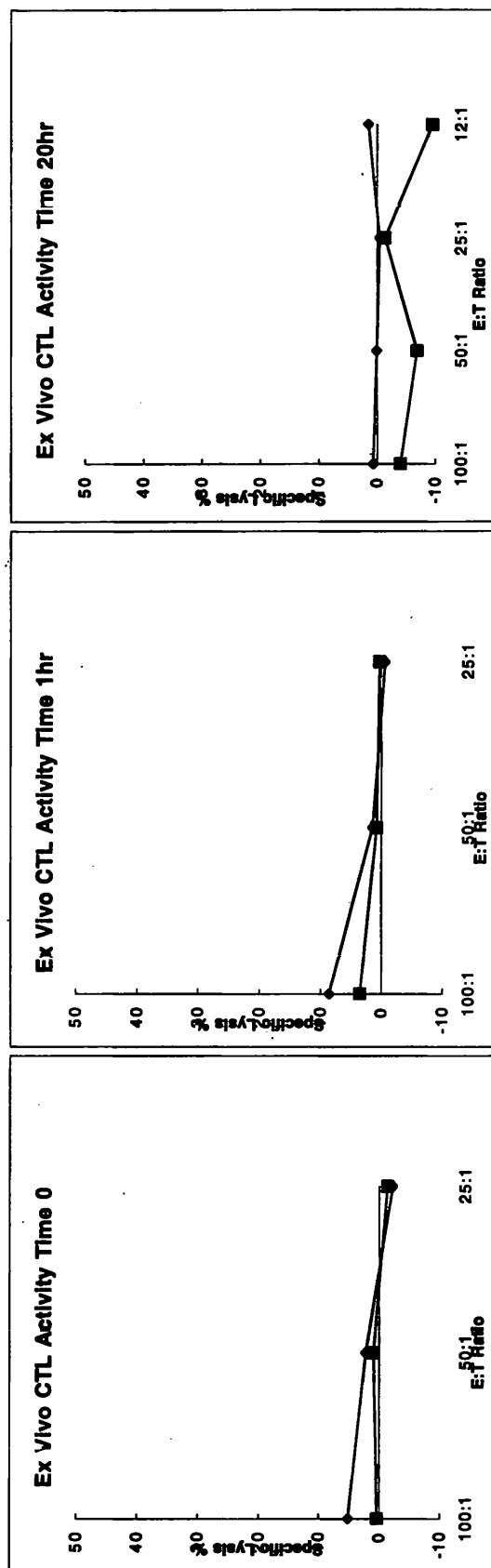
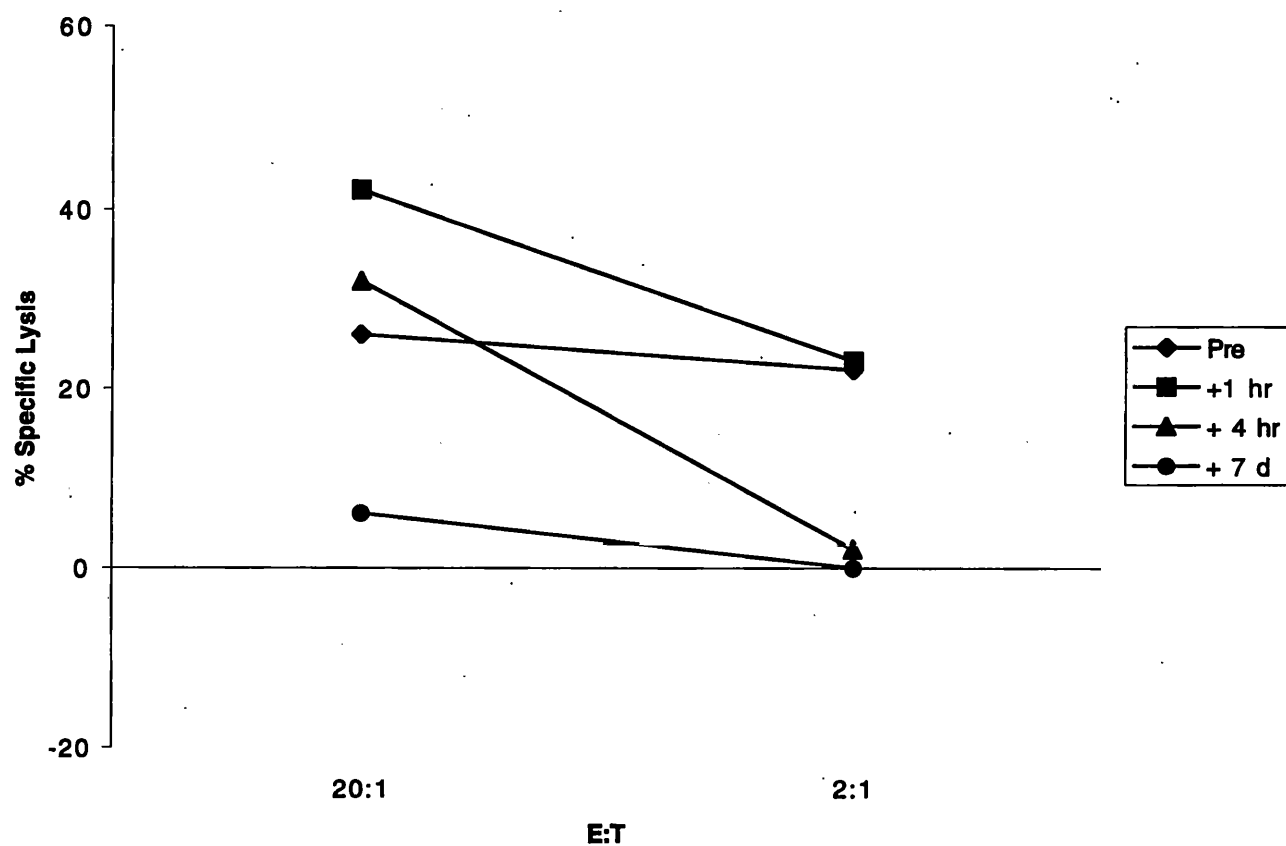


Figure 18: Patient 008 First Infusion Restimulated CTL Assays



Subsequent Infusions (Second and Third Infusions)

The lytic activity of the clones prior to the second and third infusions is shown in Figures 19 and 20. As with the first infusion, similar data were obtained for *ex vivo* and restimulated assays following the second and third infusions (Figures 21, 22 and 23) despite the increased numbers of infused CTL. No effect was seen on viral load following any infusions. Following the second infusion, PBMC were stained for V-beta 16 (the clonal TCR on the infused Clone 28) and an irrelevant V-beta 17. In accordance with the number of cells infused, the proportion of V-beta 16 positive cells rose from 1.8% to 2.4% while the proportion of V-beta 17 cells remained at 5.4%.

Figure 19: Patient 008 Clone 18 CTL activity pre-second infusion

Four cryopreserved vials of Clone 18 were thawed, restimulated and tested for cytotoxic activity with autologous B cells coated with 1 μ M peptide (GGKKKYKL).

Figure 20: Patient 008 clones pre-third infusion

Cytotoxicity of Clones 20 (circles, HLA-A2 pol), 18 (squares, HLA-B8 Gag17-3) and 28 (triangles, HLA-B8 Gag 24-13) on the day of infusion. Target cells were autologous LCL coated with 1 μ M of relevant peptide.

Figure 19: Patient 008 Clone 18

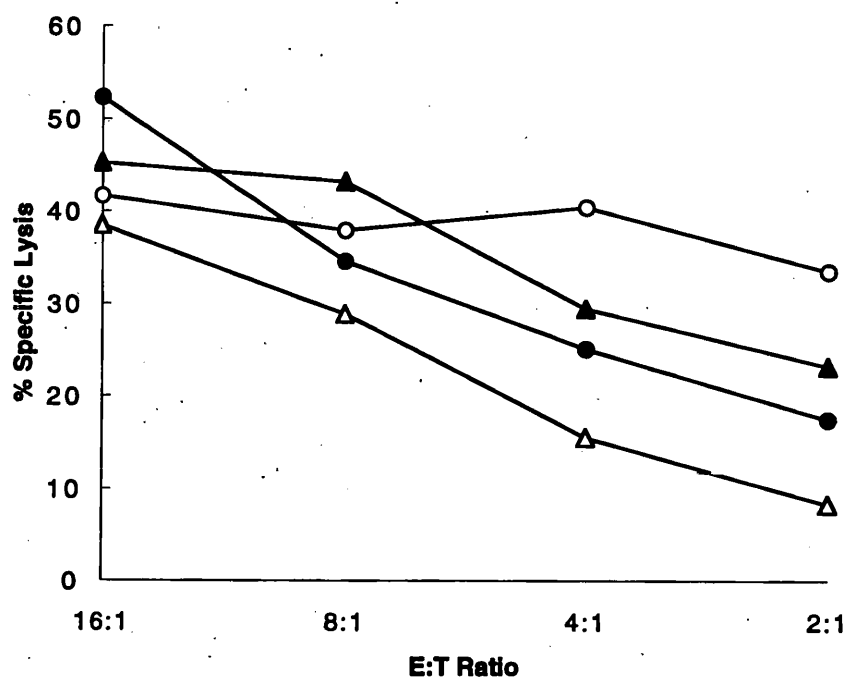


Figure 20: Patient 008 CTL Clones

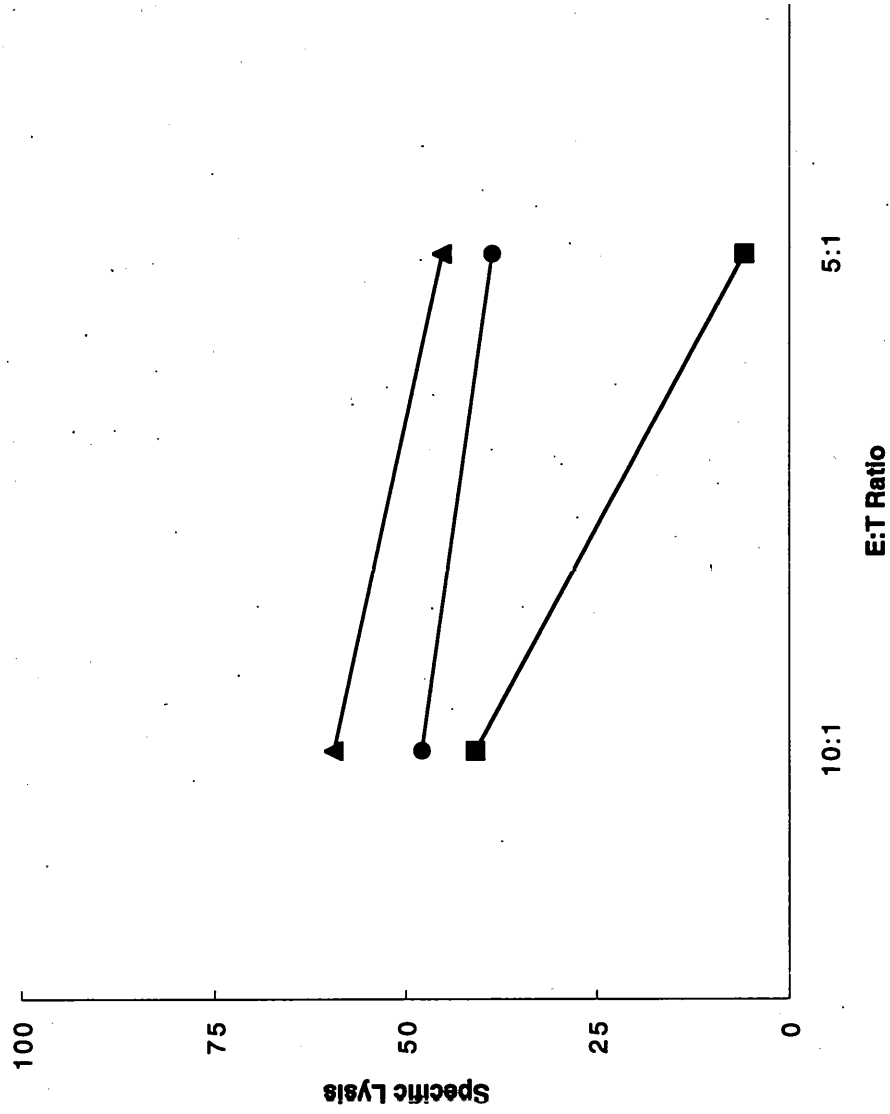


Figure 21: Ex vivo CTL activity post-second infusion

PBMC taken from Patient 008 before transfer (0h) and at 1 hr, 20 hrs and 4 days after transfer were tested immediately *ex vivo* with autologous B-cell targets coated with Gag p24-13 (diamonds) or Gag p17-3 (squares).

Figure 22: Restimulated CTL activity post-second infusion

PBMC taken from Patient 008 before transfer and at 1 hr, 4 hrs and 14 days after transfer were restimulated with Gag p24-13 (diamonds) or Gag p17-3 (squares) and tested after 14 days of culture medium containing IL-2.

Figure 23: Ex vivo and restimulated CTL activity post-third infusion

PBMC taken from Patient 008 before transfer (0hr) and at 2 hours and 6 days after transfer were tested immediately *ex vivo* with autologous B-cell targets coated with Gag p24-13 (circles), Gag p17-3 (squares) or Pol (triangles). PBMC taken from Patient 008 before transfer and at 2 hours after transfer were stimulated Gag p24-13 (circles), Gag p17-3 (squares) or Pol (triangles) and tested after 14 days of culture in medium containing IL-2.

Figure 21: Patient 008 Second Infusion Ex Vivo CTL Assays

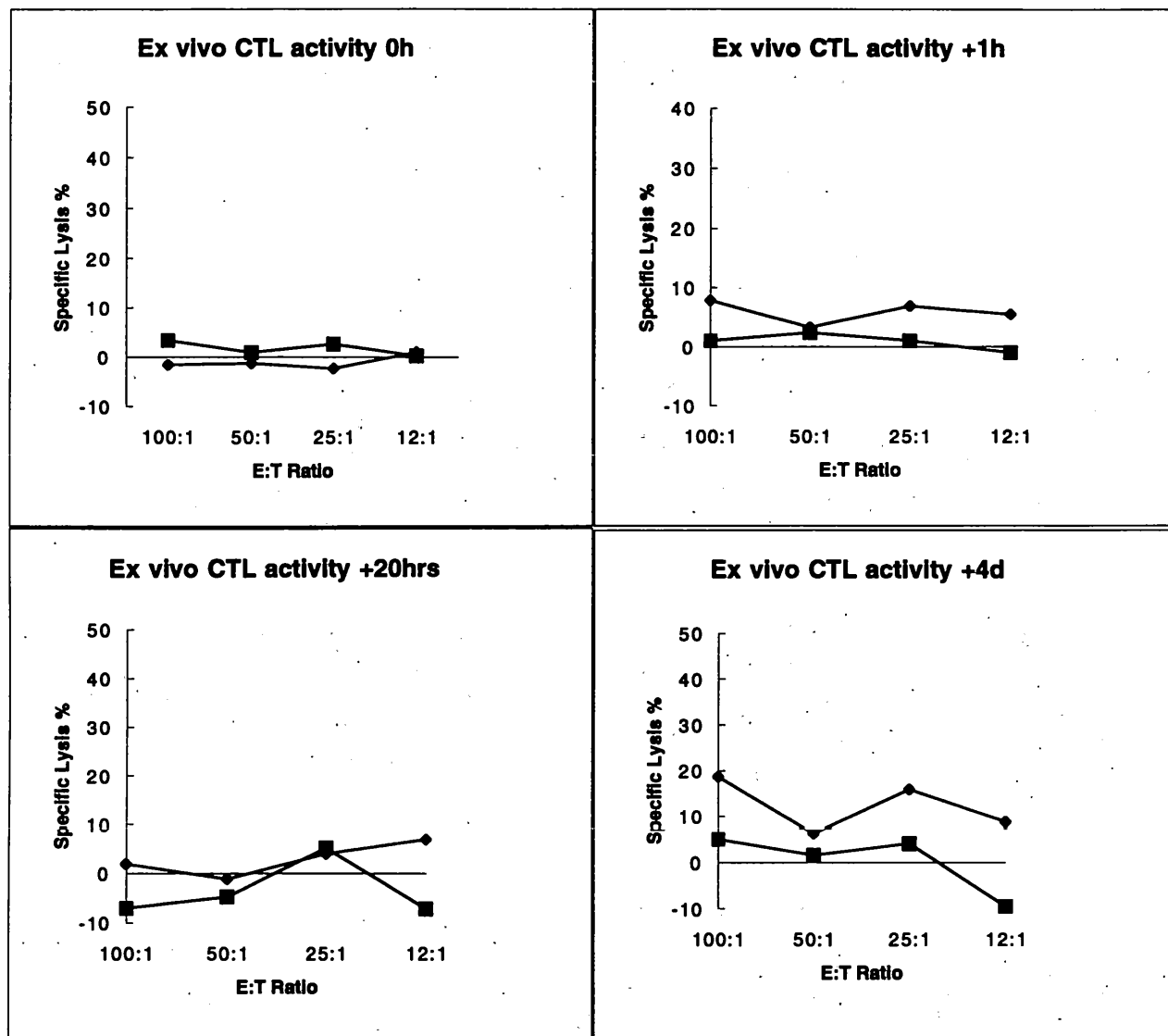


Figure 22: Patient 008 Second Infusion Restimulated CTL Assays

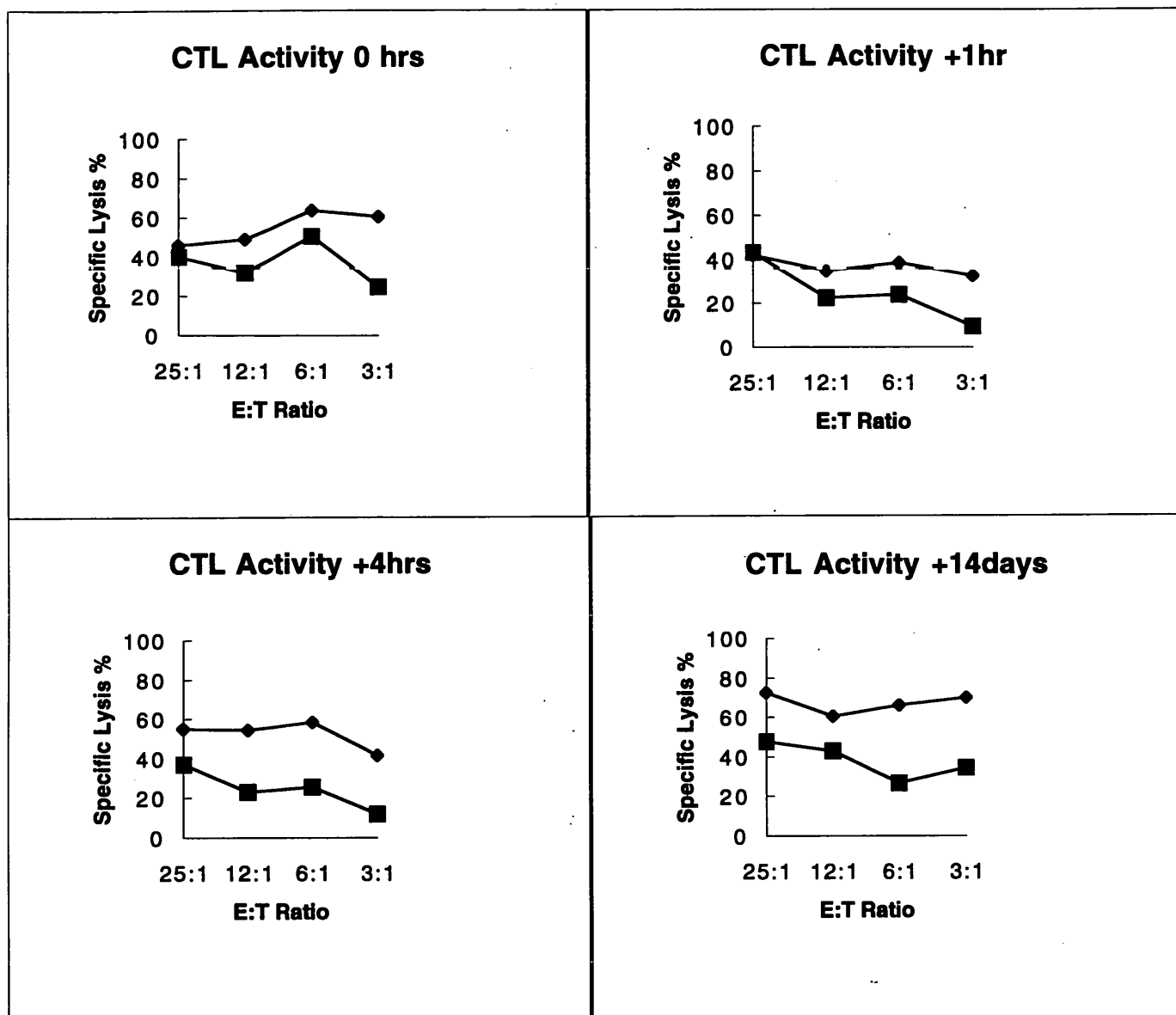
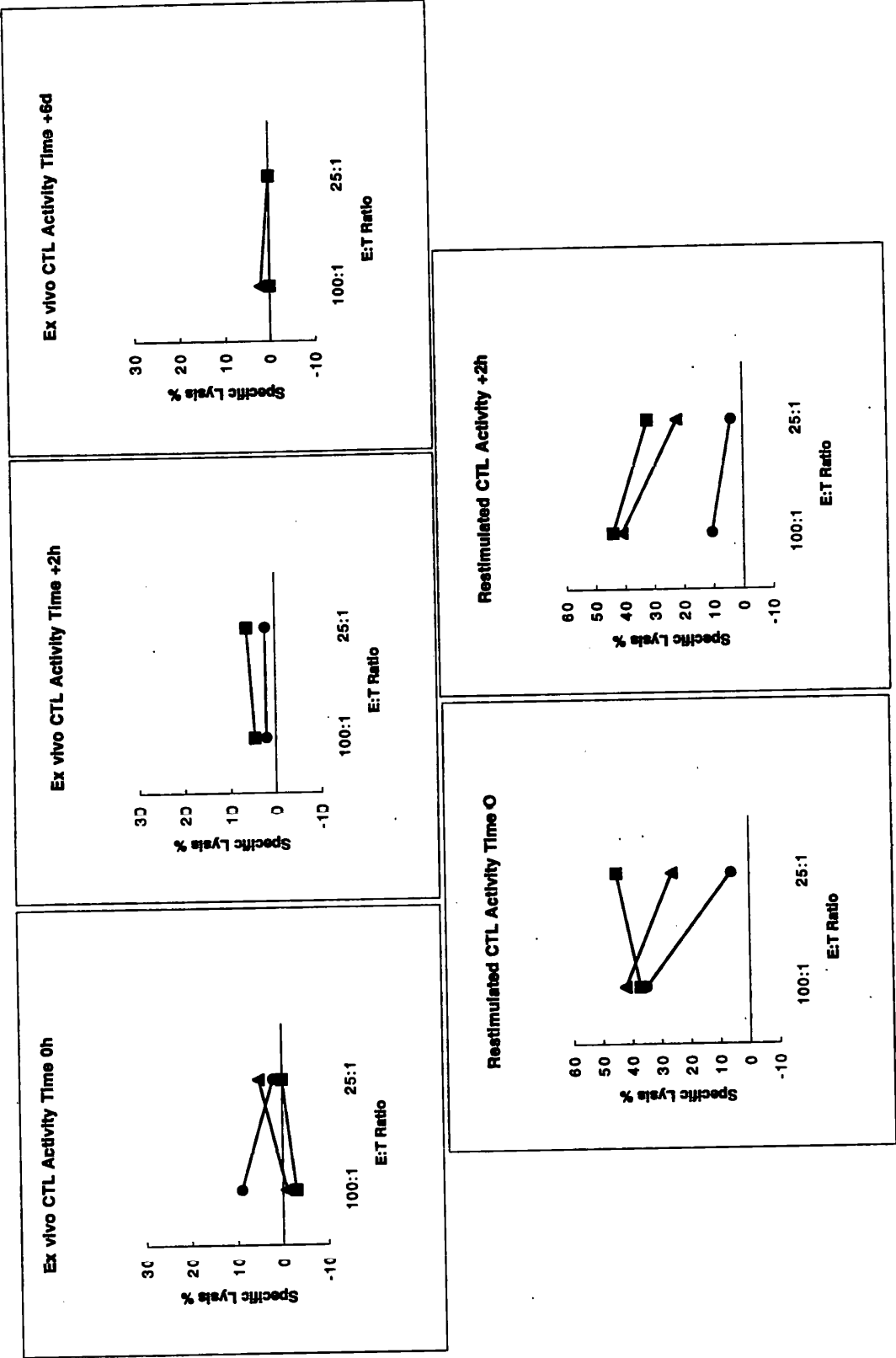


Figure 23: Patient 008 Third Infusion CTL Assays



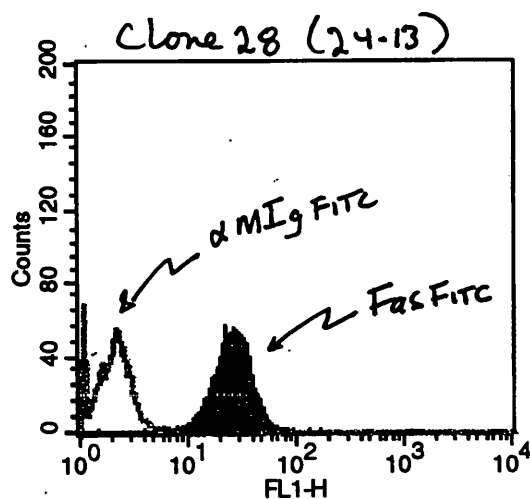
Expression of Fas

Control of lymphocyte populations, including CTL is dependent on the regulated expression of Fas and T-cell activation results in the upregulated expression of both Fas and Fas ligand. To confirm expression of Fas, Clone 28 was stained with mouse anti-Fas FITC (Figure 24).

Fas-Mediated Apoptosis

Because Clone 28 expressed Fas we were interested in determining if the clone was sensitive to Fas-mediated death *in vitro*. Clone 28 was incubated with an agonistic monoclonal antibody specific for Fas and cell death was assayed by trypan blue staining. CTL incubated with antibody were triggered to undergo apoptosis and this was demonstrated to be dependent on the expression of Fas as addition of soluble Fas fusion protein was able to prevent cell death (Table 12). Addition of IL-2 to the medium also appeared to prevent apoptosis of CTL.

Figure 24: Fas expression by Clone 28



Clone 28 was removed from cell culture and stained with anti-human Fas monoclonal antibody conjugated to FITC or mouse isotype control conjugated to FITC

Table 12: Fas-mediated death of Clone 28

| | With IL-2 | | Without IL-2 | |
|----------------------------------|----------------|------------------|------------------|--|
| | Anti Fas alone | % Live % Dead | % Live % Dead | |
| | 100 ng/ml | 88 13 | 50 50 | |
| | 50 ng/ml | 100 0 | 56 44 | |
| | 10 ng/ml | 100 0 | 43 57 | |
| Anti-Fas + Fas-antagonist | | | | |
| | 100 ng/ml | 100 0 | 77 23 | |
| | 50 ng/ml | 81 19 | 92 8 | |
| | 10 ng/ml | 93 7 | 86 14 | |

Clone 28 (HLA B8-restricted) was incubated with anti-Fas antibody (agonist) alone or with Fas-antagonist (Fas fusion protein) in the presence or absence of human IL-2 (25 cetus units/ml). Viable cells were determined by trypan blue exclusion after 16 hours.

Discussion

The results above show clearly that fastidious planning is necessary for the successful implementation of adoptive transfer. CTL clones must be developed which possess good lytic activity and good growth potential. The clones must be carefully characterized to ensure they are what they seem to be. The clones must be grown in an absolutely sterile environment and tested carefully (although retrospectively) for bacterial, fungal or viral contamination.

Perhaps the most difficult aspect of this form of therapy is the expansion of CTL clones. Previous methods employed in our laboratory, while sufficient to grow millions or even tens of millions of cells were inadequate for the large scale culture which was necessary. Clearly, the expansion of clones in plates is not practical from the point of view of labour and also the risk of contamination. Growing clones in flasks represents a good alternative. Cells appeared to grow optimally in T25 (25 ml) flasks, less well in T75 (200 ml) flasks and even less well in (600 ml) flasks. Because the maximal concentration of cells is generally around 1-2 million per ml, a single T25 flask containing 30 mls of medium will contain at most 60 million cells, generally less. Therefore, to harvest 1000 million (10^9) cells it would be necessary to use around 30 flasks for each clone. A good compromise is to grow the cells in T75 flasks which yield close to 1 million cells per ml and contain 60 mls of medium. Thus, one need only manipulate around 15-20 flasks per clone. As can be seen both from the above data and from subsequent cell growth data presented in the next chapter, there is a learning curve associated with mass cell expansion. Although the methods used from one infusion to the next did not vary, the success with growing cells certainly did.

One aspect of cell growth which may be very important, is the dividing potential of the clone. The clones used for these experiments were first made in 1991 and had undergone an estimated 5-10 cell expansions in that time. The number of actual cell divisions may be much higher. Both anecdotal data from S. Riddell (personal communication) and my own observations, suggest that ideally, one should utilize

"fresh" or "fresh-frozen" clones for large scale expansion. That is, one should clone CTL from the patients PBMC as early as possible, expand the clone once and cryopreserve many aliquots of this original expansion for subsequent expansion. These aliquots will only have been exposed to MHC-peptide or anti-CD3 twice since being removed from the circulation.

Of course, this does not take into account the fact that these antigen-specific clones, likely memory cells have been undergoing a massive cycles of mitosis *in vivo* as a result of exposure to HIV infected target cells. Nonetheless, "new clones" seem to grow better than "old clones". Perhaps physiological activation of the TCR is less damaging to the cells reproductive cycle than *ex vivo* (over)-stimulation using non-physiological doses of TCR ligand.

One mechanism by which CD8 cells may lose their divisive capacity is by the shortening of their telomeric repeats. All nucleated cells contain chromosomes, the ends (telomeres) of which are composed of hexameric nucleotide repeats. These repeating sequences are necessary for RNA initiated DNA replication during mitosis (Blackburn 1991). With each subsequent nuclear division, these hexameric repeats are lost and remain so unless the ends are re-synthesized by the enzyme, telomerase. I have looked at the telomere length of clone 28 retrospectively. Unfortunately, the proper control which would be clones freshly isolated from Patient 008, were not available. Thus I compared the telomere length of clone 28 to that obtained from three laboratory volunteers. The telomere lengths visualized were 11-12 kilobases and did not differ between Clone 28 and the three volunteers (all similar in age to Patient 008).

If telomere length is actually shortened significantly in the CTL clones, this implies not only that they may be difficult to expand *ex vivo*, but that they may have a limited efficacy *in vivo* following adoptive transfer. Ideally, the transferred memory cells would expand upon contact with antigen and carry out their effector function

over many cycles of replication. This may not be the case with cells that have been over-expanded.

Another factor which may determine the efficiency and efficacy of the clones *in vivo* is the cytokine milieu and lymphoid architecture *in vivo*. As discussed in the introduction, the bulk of HIV-infected cells reside in the reticulo-endothelial organs, in particular the lymph nodes. In the lymph nodes, the lack of CD4 cells, quite pronounced in a patient with a CD4 count of 20/ μ l may result in a physiological lack of IL-2. Without IL-2 the transferred cells may not survive. In addition the architecture of the lymph nodes in advanced HIV disease is generally aberrant perhaps preventing proper lymphocyte re-population or preventing normal lymphocyte-lymphocyte contact.

It may not be coincidental that thus far, the successful use of adoptive CTL transfer in humans has been in recipients who had been preconditioned either with radiation or with chemotherapy. Ablation of dividing cells may produce an environment more receptive to repopulation of infused cells. Certainly, adoptive transfer experiments conducted in mice and rats and published many years before human adoptive transfer frequently raised the concept of "space" ((Makela and Mitchison 1965, Makela and Mitchison 1965); Cocharne et al, 1962; Dixon et al, 1963) as a necessary precondition for optimal adoptive therapy. Space was generally created by prior irradiation of the recipient animal. This was hypothesized to produce a depletion of lymphocytes in the peripheral lymphoid organs which could then be more readily replaced by transferred cells. Alternatively, Celada has argued that a natural barrier to transferred cells exists and that irradiation may remove this barrier (Celada 1967). Bell et al transferred thoracic duct lymphocytes with a memory T-cell response to HSA into both normal and irradiated congenic rats (Bell and Shand 1975). In one experiment, they removed lymphocytes from potential recipients through a thoracic duct fistula in an attempt to create cellular space in a non-irradiated host. By measuring HSA antibody response, they found that the lymphocyte depleted recipients had no greater antibody responses than normal

recipient rats. In contrast, irradiated recipients had one to two log increases in antibody responses when compared to normal rats. Moreover, by comparing adoptive therapy of similar cells into young (3 week old) rats and comparing them to older (6 month old) rats, they showed that young recipients had antibody responses greater than normal older rats and in the range of irradiated recipients. Thus, there seems to be a factor present in older animals which limits reconstitution by infused memory cells. This is partially supported by the experiences of bone marrow transplantation where younger recipients generally fare better.

Hematopoietic stem cells, referred to in stem cell therapy as colony forming units are pluripotent cells which are able to reconstitute the bone marrow of a preconditioned host. Memory cells, in contrast are mature, differentiated cells which have undergone numerous cell divisions *in vivo* and in the case of adoptive immunotherapy, *ex vivo*. Thus it is somewhat surprising that peripheral CD4+ lymphocytes with a memory phenotype have been shown to be capable of dividing up to 56 times *in vivo* following transfer into nude mice, a number of potential divisions similar to that of stem cells (Rocha, et al. 1989). However, the replicative potential of CD8+ cells appears to be lower and estimates are that they are able to double up to 17 times after transfer (Miller and Stutman 1984).

The replicative ability of adoptively transferred lymphocytes has been examined many times. Miller et al transferred limiting numbers of splenic cells into mice which had been thymectomized, lethally irradiated and reconstituted with T cell-depleted bone marrow. In this model, repopulation of helper and cytotoxic T-cells occurred up to 20% of normal although these cells appeared to be sufficient to protect animals from infection and behaved normally in both CTL and proliferation assays. Similarly, nude rats reconstituted with syngeneic thoracic duct lymphocytes exhibited a stable and permanent expansion of functional T-cells for at least 2 years following transfer (Bell 1979). It is worth noting however, that in most experiments, adoptive transfer of undepleted spleen cells was done. Thus, it is still unclear whether a co-transfer of CD4+ cells is necessary to positively influence the growth

of CTL. In patients with advanced HIV, this may be a critical point, as there may be insufficient IL-2 production by remaining CD4+ cells or indeed insufficient repertoire of CD4+ cells to maintain growth of infused CTL.

In addition, the nature of the transferred cells may also influence its efficacy *in vivo*. Berzofsky has shown that cells cloned using a high concentration of MHC-peptide have a lower avidity for target cells and compare unfavourably with high avidity cells in their ability to clear a virus infection in mice. In later cloning experiments, I lowered the peptide concentration for cloning from 100 μ M to 1 μ M or less and found empirically that the killer activity of the clones was improved. *In vitro*, the peptide concentration on the surface of the cells may be several orders of magnitude lower than the concentration on target cells used for *in vitro* chromium release assays. Cloning and culturing CTL using high concentrations of peptide may select for relatively low affinity clones which may not possess significant *in vivo* activity.

Finally, the expression of Fas on the CTL clones may provide another means by which HIV-infected cells are protected. We have described a phenomenon known as "back-killing" by which SIV-infected cells, instead of behaving as passive target cells, actually kill incoming CTL by engaging Fas on their cell surface (Xu, et al. 1997). HIV infected cells also express Fas ligand and most of the CTL clones which I have tested express Fas. It is possible that when the HIV-specific CTL home in on their Fas-ligand expressing targets, they are activated to undergo apoptosis. In particular, since IL-2 appears to protect lymphocytes from undergoing apoptosis, the lack of CD4 cells in this patient may render the CTL more susceptible to back-killing. We have attempted to address this mechanism by a simple *in vitro* experiment. Clone 28 which expresses Fas (Figure 24) and was infused into Patient 008 was washed and placed in R10 without IL-2. Timing of the experiment is important because clones do not survive for more than a few days without IL-2. Cells were placed in 24 well plates containing only R10 or R10 plus a Fas agonist or R10 plus a Fas agonist and Fas antagonist. The results in Table 12 show that anti-Fas can indeed kill

the Fas-expressing clone. Moreover, this killing is specific since it is blocked by the Fas antagonist and the clone is protected from apoptosis in the presence of IL-2.

In summary, there are two major obstacles to overcome in order for adoptive transfer therapy to become successful. These include developing the necessary infrastructure and two, understanding the complex biology of CTL-target interaction *in vivo*. In the case of HIV infection, the multitude of possible explanations for the inefficacy of adoptive transfer need to be examined and either refuted or endorsed. In the next chapter, we hope to shed further light on these mechanisms.

CHAPTER FOUR

Adoptive Transfer Of Autologous HIV-Specific CTL To A Patient With A Relatively High CD4 Count

Introduction

In Chapter One we discussed the successful use of adoptive CTL immunotherapy for the treatment of EBV and CMV disease in post-BMT patients (Riddell, et al. 1992, Rooney, et al. 1995, Walter, et al. 1995), thus raising the possibility that treating HIV-infected patients in a similar fashion may be beneficial . However, the transfer of HIV-specific CTL lines or clones in HIV-infected patients, although safe, has so far produced only modest results (Koenig, et al. 1995, Lieberman, et al. 1997, Riddell, et al. 1996).

We have seen in Chapter Three that adoptive transfer of HIV-specific CTL to Patient 008, who had a CD4 count of around 20/ul and a relatively low virus load, also failed to impact upon virus load. As discussed in that chapter, there are a number of theoretical reasons why the advanced state of his disease may have affected the efficacy of the therapy. In summary, the three main arguments are:

1. A low CD4 count may hamper optimal CTL effector function because IL-2 is necessary both as a growth factor for CTL and for optimal CTL effector function. Thus, the presumed lack of physiological levels of IL-2 in the lymph nodes of a patient with advanced HIV, may prevent adoptively transferred cells from dividing, maturing and lysing infected targets.
2. Upon meeting infected cells, CTL may either form an MHC-peptide-TCR complex or a FasL/Fas bond resulting in target cell lysis. In addition, the two cells may form a reverse Fas-FasL bond (FasL on the target cell) triggering apoptosis of

the CTL. As shown in Table 12, lack of IL-2 may predispose CTL to death by apoptosis and conversely, presence of IL-2 protects CTL from Fas-mediated death.

3. Finally, virus load may also play a role in stimulating the CTL to divide and respond. A relatively low virus load may lack sufficient quantities of antigen to activate CTL optimally. Thus, in a patient with a high CD4 count and a relatively high (and increasing) virus load, the adoptive transfer of autologous HIV-specific CTL may be more effective.

In this chapter we present data from a single infusion of HIV-specific CTL into a patient with a relatively high CD4 count and a relatively high virus load. Thus we have the opportunity to examine the effect of transferred cells in a milieu of high CD4 counts and possibly physiological levels of IL-2 production. In addition we examine the discrepancy between results obtained in human adoptive transfer experiments for EBV associated lymphoproliferative disease and the failure of transferred HIV-specific CTL to significantly reduce virus loads.

We have transferred two autologous CTL clones specific for epitopes derived from Gag and Pol to a patient with a high virus load and clinical disease. In order to trace the fate of the infused cells *in vivo*, we have used soluble MHC-peptide molecules (tetramers) specific for the TCR of one of the infused clones.

Methods

Study patient

Patient 868 is a white homosexual male (HLA-A*0201,A*2401,B*27,B*3501) who, upon recruitment, had been HIV positive for over 6 years. Despite previous episodes of *Pneumocystis carinii* pneumonia and recurrent Herpes simplex infections, at the time of transfer, he was well and his CD4 count was 430/μl. Despite this relatively high CD4+ count the patient was experiencing symptoms

such as fatigue and sweats which were attributed to high viral load and had only partially been controlled by antiretroviral drugs. His medications included zidovudine, didanosine and prophylactic trimethoprim-sulfamethoxazole. Ethical approval was obtained from Central Oxford Regional Ethics Committee and informed consent from the patient.

Preparation and expansion of CTL clones

In contrast to Patient 008, whose results are described in Chapter 3, we were able to prospectively generate CTL clones from Patient 868. In theory, these clones should be more efficient than clones which were resuscitated from frozen stocks. CTL oligoclonal lines were produced by incubating fresh PBMC obtained from the patient with irradiated, autologous EBV-transformed lymphoblastoid cell lines (LCL) coated with peptides derived from Gag and Pol. Two CTL lines with lytic activity to HLA A*0201-restricted epitopes from Gag (p17 residues 77-85, SLYNTVATL) and Pol (residues 346-354, VIYQYMDDL) were generated and used for establishing clones by limiting dilution. Large scale expansion of CTL is described in detail in Chapter 3. CTL were grown in T25 or T75 tissue culture flasks, each containing the T-cell clone, irradiated allogeneic PBMC and irradiated allogeneic LCL. TCR stimulation was provided by monoclonal anti-CD3 (OKT3, a kind gift from Orthoclone) and the culture medium was RPMI1640 supplemented with 10% human AB serum, glutamine, penicillin and streptomycin. Cells were harvested and washed the day following setup and resuspended in medium containing recombinant IL-2 (50 cetus units/ml). The cells were divided periodically according to growth and harvested 12 days after TCR stimulation. Aliquots of cells and supernatants were sent for extensive microbiological testing to ensure sterility.

Cytotoxicity assays

Chromium-51 (Cr-51) release assays were as described. Autologous LCL were incubated with Cr-51, pulsed with 5 μ M peptide and incubated with CTL. Supernatants were harvested after 4-5 hours and measured in a betaplate counter (Wallac).

Anchored PCR

The anchored PCR method has been described previously. Oligo dT-primed cDNA was synthesized from RNA obtained from two million T cell. The primers used were, Poly-C anchor - 5'-gcattcagctgcggccgc(c)14-3'; c β 1-5'-tgaccgcagtcgacagactgtcactggatt-3'; c β 2 5'-atactggagtcgacggagatctctgcttctgatg-3'.

Soluble MHC-peptide tetramers

MHC-peptide tetrameric complexes were produced as previously described. Purified HLA heavy chain and β -2m were synthesized using a bacterial expression system (pET R+D Systems). The heavy chain was modified by the deletion of the transmembrane/cytosolic tail and the addition of a C-terminal sequence containing the BirA enzymatic biotinylation site (anti-sense primer for HLA-A*0201

5'gggggaagcttaatgccattcgatttctgagcttcaaaaatatcgttcataccaccaccggatccacatacaagcttaggctcccatcttaaggtgaggggcttggg3'. Heavy chain, β 2 microglobulin and peptide were refolded by dilution. The 45kD refolded product was isolated by FPLC, and then biotinylated by BirA in the presence of biotin (Sigma), ATP (Sigma) and Mg^{2+} (Sigma). Streptavidin-phycoerythrin conjugate (Leinco) was added in a 1:4 molar ratio and the tetrameric product was concentrated to 1mg/ml.

Flow cytometry , monoclonal antibodies and virus loads

The CTL clones were phenotyped by dual-staining with PE-conjugated anti-CD8 mAb (Dako) and FITC-conjugated anti-CD3 (Dako), anti-CD28 (Immunotech) or anti-Fas mAb. Labeled cells were analysed on a Becton Dickinson FACScan using Cellquest software. Negative isotype control mAbs were FITC- and PE-conjugated mAbs of irrelevant specificity (Dako). CD4 and CD8 cell counts were done in a clinical immunology laboratory by standard methods. The HIV plasma concentrations were determined using a commercial (Roche Amplicor) PCR assay.

Apoptosis assay

To analyse apoptosis *in vivo*, PBMC obtained before or after infusion were stained with 3 μ l of PE-conjugated A2 Gag-specific tetramer (1 mg/ml) at 4 °C for 30 minutes. After two washes the cells were stained with 100 μ l Annexin-V-Fluos (Boehringer Mannheim) for 15 min. To test whether the CTL clones were sensitive to FasL-induced cell death *in vitro*, 5×10^5 CTL were co-cultured with a FasL expressing cell line (1×10^6 cells, 1A12, a gift from Dr. S. Nagata, Osaka) with or without soluble Fas-Fc fusion protein (20ug/ml) in a 48-well plate for 16 hours. Cells were dual-labeled with PE-conjugated CD8 and Annexin-V-Fluos and analysed by flow cytometry as described above.

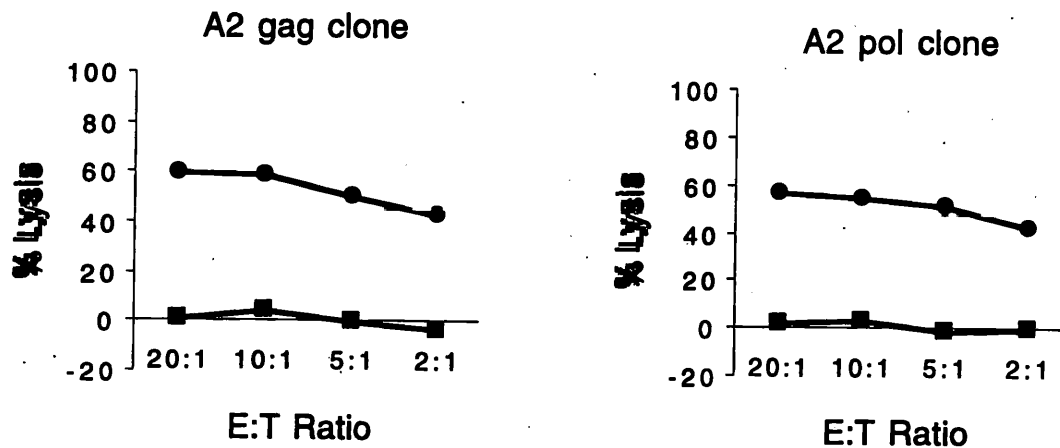
Results

HIV-specific clones

Two HLA A*0201 restricted CTL clones were established from Patient 868. The first recognizes the immunodominant HLA A*0201 restricted epitope from Gag p17-8, SLYNTVATL (Figure 25a) and the other recognises VIYQYMDDL an uncommonly seen epitope derived from Pol (Harrer et al). This particular epitope is interesting because it contains the active site (YMDD) of the HIV reverse transcriptase (Figure 25b) and any mutations affecting this active site should abrogate the replicative capacity of the virus. CTL responses to this particular epitope are much less common than for the "immunodominant" Pol epitope, ILKEPVHGV (Table 13). Peptide titration studies of the A2 Pol clone revealed cytotoxicity only in the presence of large concentrations of peptide (Figure 26) – at 1 μ M of peptide, most of the specific lytic activity is lost. This may account for the fact that CTL to this epitope are infrequently detected.

The activity of the A2 Pol clone was also tested against a number of peptide variants described in the literature (Table 13). In contrast to Harrer et al, our clone

Figure 25: Patient 868 CTL Clones for Adoptive Transfer



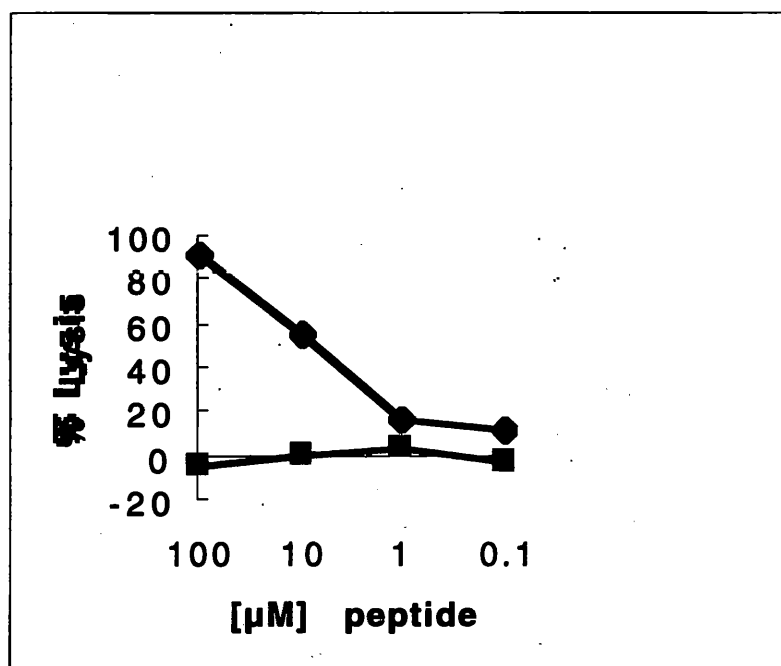
The lytic activity of the A2 Gag and A2 Pol clones on the day of infusion. CTL were tested against autologous target cells coated with SLYNTVATL (circles, left panel) or VIYQYMDDL (circles, right panel). Squares indicate uncoated target cells.

Table 13: CTL responses to HLA-A*0201 restricted reverse transcriptase epitope

| | P | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | | |
|----------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------------|--------|
| | RT | 179 | 180 | 181 | 182 | 183 | 184 | 185 | 186 | 187 | 188 | Harrer et al | Thesis |
| HIV-1 IIIB | V | I | Y | Q | Y | M | D | D | L | | | ++ | ++ |
| P1 variant | T | I | Y | Q | Y | M | D | D | L | | | ++ | na |
| P1 variant | I | I | Y | Q | Y | M | D | D | L | | | ++ | na |
| P1 variant | E | I | I | Q | Y | M | D | D | I | L | - | | na |
| HIV-2 Rod | I | I | I | Q | Y | M | D | D | I | L | + | | - |
| HIV-2 Rod | I | L | I | Q | Y | M | D | D | I | L | na | | - |
| HTLV-1 | T | I | L | Q | Y | M | D | D | I | L | - | | na |
| HTLV-2 | T | I | V | Q | Y | M | D | D | I | L | - | | na |
| SIV | T | L | V | Q | Y | M | D | D | L | | - | | - |
| Lamivudine/3TC | V | I | Y | Q | Y | V | D | D | L | | - | | na |
| Nevirapine | V | I | C | Q | Y | M | D | D | L | | - | | - |

The index peptide (VIYQYMDDL) from HIV reverse transcriptase (RT) and variants as listed above were tested for cytotoxic activity using the Patient 868 A2 Pol-restricted CTL clone and the results were compared with previously published results using RT-specific clones (Harrer et al). ++ refers to > 50% specific lysis, + refers to 20-50% specific lysis and - refers to <10% specific lysis.

Figure 26: Peptide titration of A2 Pol clone



The lytic activity of Patient 868 derived A2 Pol CTL clone was assayed against peptide coated autologous LCL target cells incubated with decreasing concentrations of peptide (diamonds) or target cells with no peptide labelling (squares).

does not recognize the P1 variants. In addition, antagonism was not demonstrated using variant peptides, TLVYQMDDI, VICQYMDDL, ILIYMDDI or Pol index peptide, ILKEPVHGV.

Both the A2 Gag clone and the A2 Pol clone expressed CD3, CD8 and Fas, but not CD28 (Figure 27). The A2 Pol clone apparently contained two populations of cells expressing CD3medium/CD8high and CD3high/CD8medium respectively. The specificity of both clones was characterized using soluble, biotinylated tetrameric MHC-peptide molecules. Dual staining of the A2 Gag clone with soluble MHC-Gag tetramer and anti-CD8 demonstrated specificity for HLA A*0201-restricted Gag peptide (Figure 28a). Attempts to stain clone 10 failed because the Pol-tetramer complex was unstable, most likely a consequence of the low affinity of the Pol epitope for HLA A*0201 (Figure 26). Thus, the complementarity determining region 3 (CDR3) of the clone was sequenced by anchored PCR in order to determine its V-alpha and V-beta sequences (Figure 28b). Of 18 transcripts sequenced, all contained the same CDR3 nucleotide segment corresponding to Va22/Ja9.11 and Vb13.3/Jb2.3/Cb2 and providing evidence of clonality.

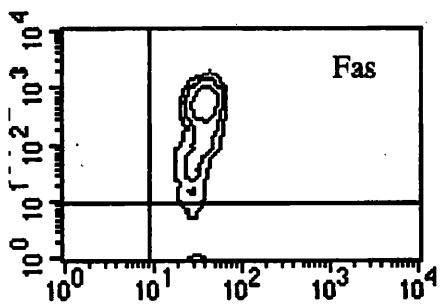
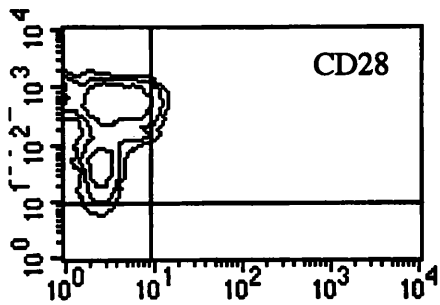
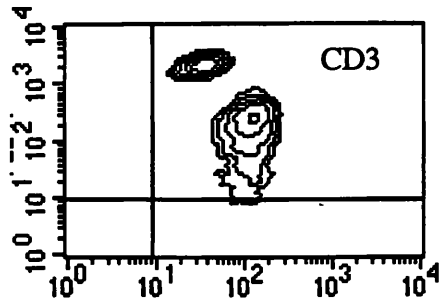
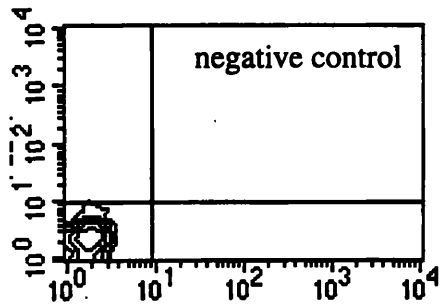
Adoptive transfer

The A2 Gag and Pol clones were grown to 1.1×10^9 and 1.7×10^9 cells respectively for adoptive transfer and the expanded clones were tested for cytotoxic activity the day before transfer (Figures 25a and 25b). The cells were infused into a peripheral vein over a period of 30 minutes. The transfer was well tolerated except for a single episode of chills and rigours approximately 4-6 hours following infusion. These subsided without the use of medications and the patient was subsequently well.

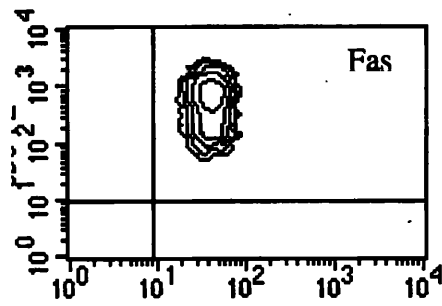
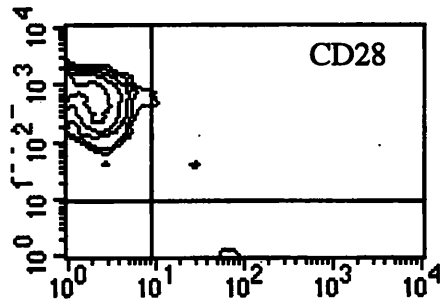
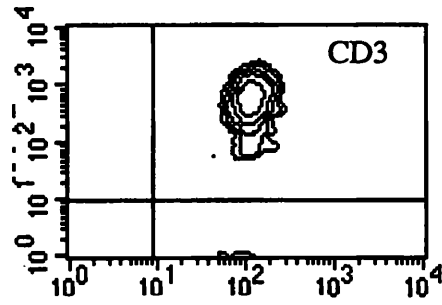
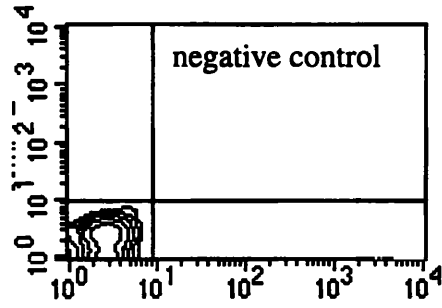
CD4/CD8 and virus loads post-infusion

Table 14 shows the peripheral blood CD4 and CD8 lymphocyte counts and the virus load before and after adoptive transfer. Unexpectedly, no increase was

A2pol clone



A2gag clone

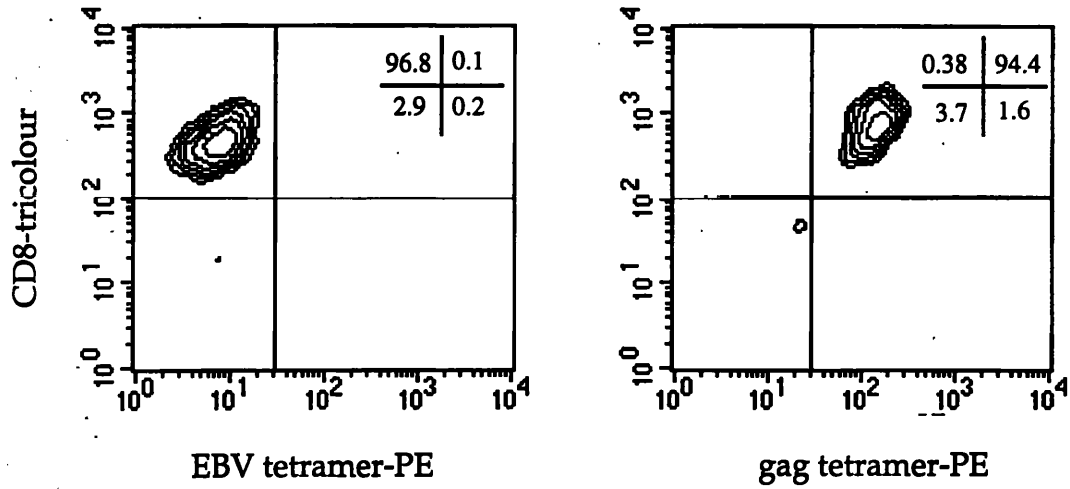


CD8-PE staining

FITC-conjugated mAb

Figure 28: Patient 868 Clone Specificity

a.



b.

| | V region | CDR3 | J region |
|----|----------------------------------|--------------|----------|
| Vα | VQVSDSAVYFCALSDNFNKFYFGSGTKLNVKP | | |
| Vβ | AAPSQTSVYFCASRF | -TDTQYFGPGTR | LTVL |

Table 14: CD4 and CD8 counts and virus load before and after infusion

| Time | 0 | +1hr | +20hr | +48hr | +7d | +14d | +21d |
|---------------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|-------------|
| CD4 (cells/μl) | 430 | 440 | 390 | 490 | 480 | 510 | 400 |
| CD8 (cells/μl) | 1720 | 1760 | 1600 | 2030 | 1910 | 2100 | 1810 |
| Virus load (RNA copies/ml) | 31,000 | 26,600 | 49,300 | 45,970 | 63,500 | 56,300 | ND |

CD4 and CD8 counts were determined by flow cytometry and plasma viral load was measured using the Roche Amplicor assay at the time points indicated.

detected in the number of CD8+ cells one hour or 20 hours after infusion. There were no significant changes in the virus load following therapy.

Tracking Clone 19 *in vivo*

PBMC obtained from the patient before and after adoptive transfer were stained with the TCR-specific tetramer (which recognizes the TCR on the A2 Gag clone) and CD8-tricolour (Figure 29). Controls included studies using a B35 tetramer complexed with an HIV Env-derived peptide, which had not been infused. Tetramer-positive cells accounted for 1.6% of CD8 cells in the patient before the infusion, increasing to 2.2% after infusion. Given that 2.5×10^5 staining events were measured at each time-point with the same reagents, this represents a real and substantial increase. This 37% increase in tetramer positive cells is approximately what would be expected assuming total body CD8+ T-cells were 2×10^{11} pre-infusion, with 3.2×10^9 pre-existing tetramer-positive cells and an infusion of 1.1×10^9 additional tetramer-positive cells. In order to determine the percentage of Gag-specific cells undergoing cell death, PBMC obtained from the patient were also triple stained with tetramer, CD8-tricolour and a marker of apoptosis, Annexin V-FITC (Figure 30). In tetramer positive cells, the proportion of dead cells increased from 29% before infusion to over 90% of cells 48 hours after infusion.

Fas ligand induced death of CTL clones

Because Clone 10 and Clone 19 expressed Fas and underwent apoptosis *in vivo*, we examined the susceptibility of the CTL to Fas-dependent apoptosis *in vitro* by incubating each clone separately with a Fas ligand expressing cell line, 1A12. Cell death was assayed by flow cytometry (Figure 31). CTL incubated with 1A12 were triggered to undergo apoptosis. This was demonstrated to be dependent on the expression of Fas as addition of soluble Fas fusion protein was able to prevent cell death.

Figure 29: Tetramer-specific A2 Gag cells before and after infusion

PBMC taken from the patient at the indicated timepoints were stained with anti-CD8 tricolour, A2 Gag-specific tetramer PE and a control, B35 Env-specific tetramer PE. The percentages shown represent CD8-positive/tetramer-positive cells.

Figure 30: Death of tetramer-specific A2 Gag cells *in vivo* before and after infusion

The A2 Gag clone (before infusion) and PBMC obtained from the patient at the timepoints indicated were stained with anti-CD8 tricolour, Gag-specific tetramer PE and Annexin V. The histograms represent Annexin V staining of the CD8-positive/tetramer-positive cells.

Figure 31: Fas-dependent death of A2 Gag clone

A2 Gag and Pol clones were incubated separately with medium alone or with a Fas-ligand expressing cell line, 1A12 with or without Fas fusion protein (FasFc). CD8 positive cells were gated and examined for Annexin V staining.

Figure 29: Patient 868 A2 gag clone post-infusion

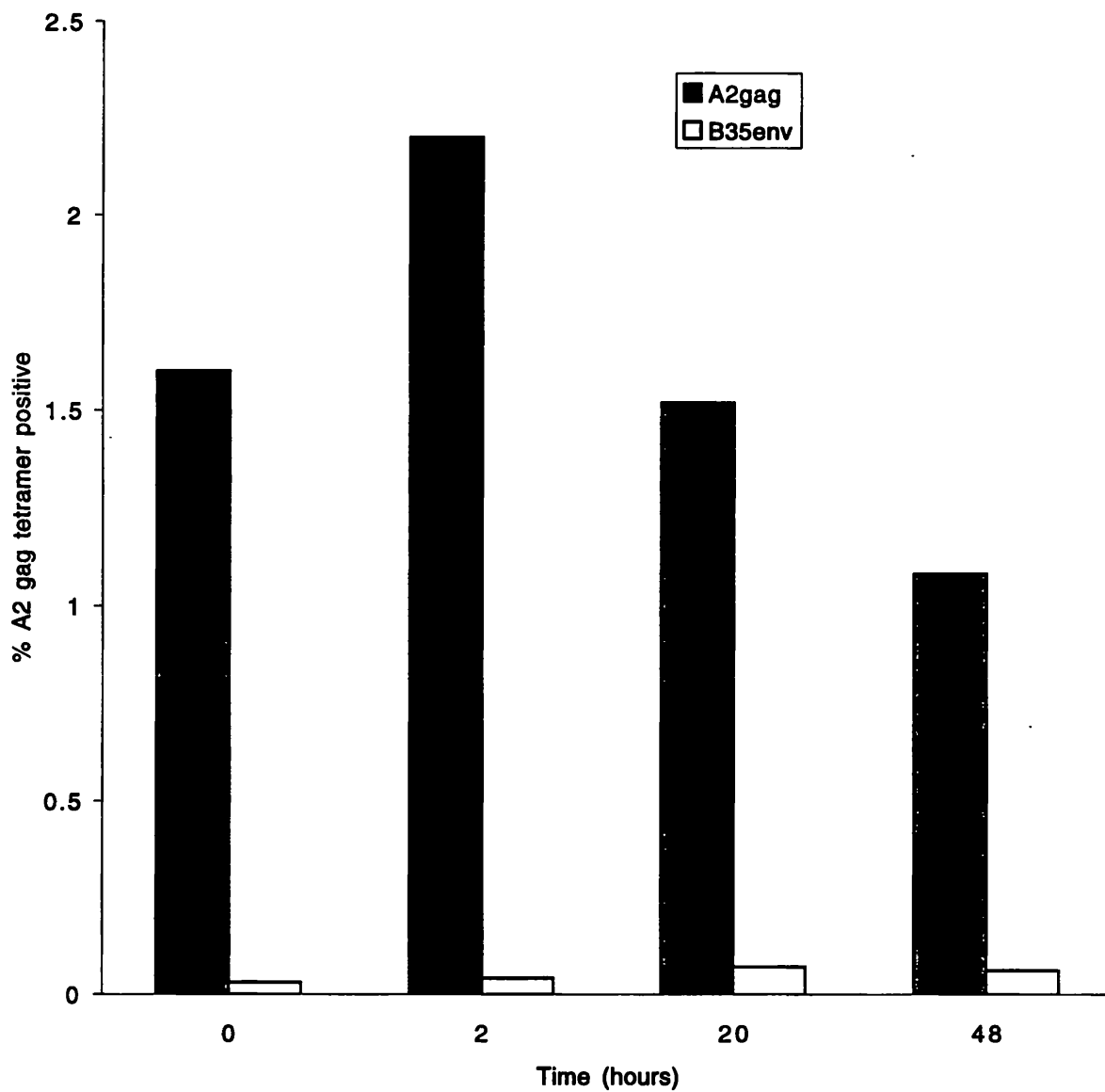
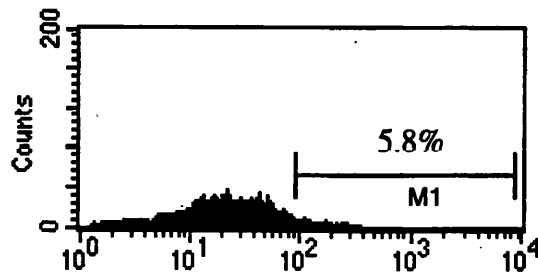
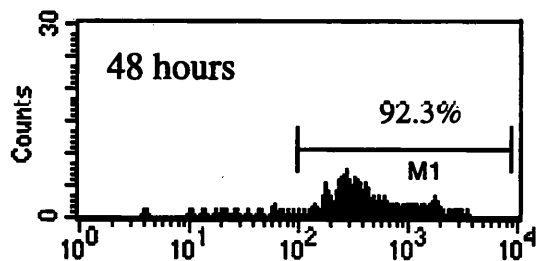
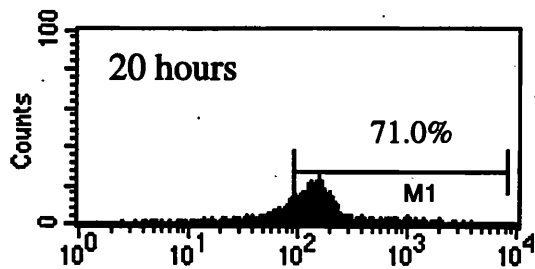
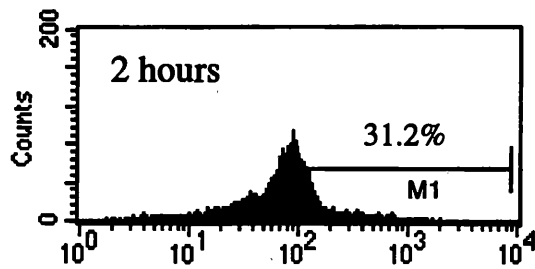
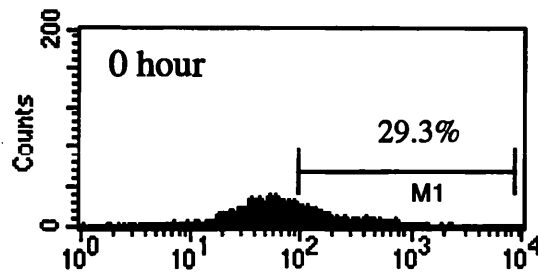


Figure 30: In vivo fate of A2 Gag Clone

A2 gag clone before infusion

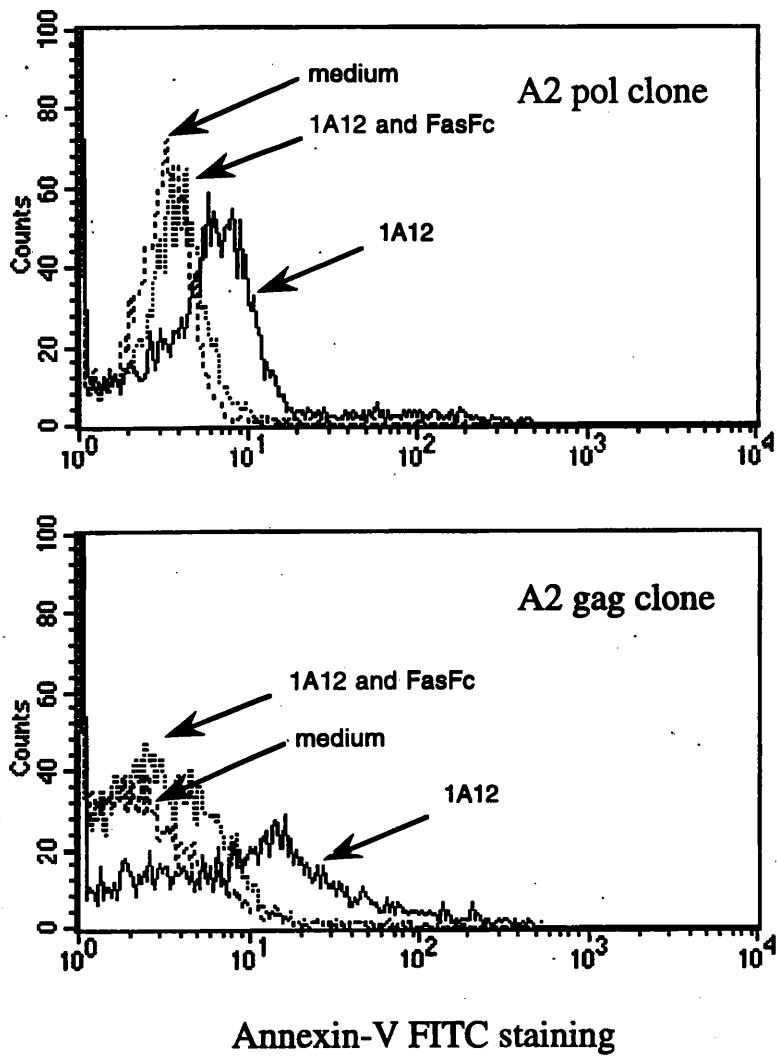


PBMC before and after infusion



Annexin-V staining

Figure 31: Patient 868 Clone Susceptibility to Fas Ligand



Discussion

The utility of adoptive transfer for the treatment and prevention of EBV and CMV infections in patients immunosuppressed following BMT (Papadopoulos, et al. 1994, Rooney, et al. 1995, Walter, et al. 1995) suggested that a similar strategy might be useful in HIV and early reports involving the infusion of non-specific, autologous CD8+ cells to HIV-infected patients showed the general method to be safe although ineffective for reducing viral replication (Ho, et al. 1993, Klimas, et al. 1994). However, the subsequent transfer of HIV-specific CTL lines and clones have produced only modest outcome or possibly detrimental outcomes (Koenig, et al. 1995, Lieberman, et al. 1997, Riddell, et al. 1996).

We have examined the *in vivo* fate of transferred CTL to explain the apparent lack of *in vivo* activity. The number of cells we infused represented around 1% of the total CD8+ cells present in the patient - a frequency at the high end of the spectrum in HIV-positive subjects and one that would be expected to reduce viral replication measurably. However, despite attempts to optimise therapy, the infusions failed to reduce virus load. In an attempt to explain the lack of efficacy, we traced the infused cells using soluble, tetrameric HLA A*0201-Gag molecules, which are able to stain specifically the infused Gag clone by binding to its clonotypic TCR. A short-lived increase was seen in the clonal cells following infusion, with the number of tetramer-positive CD8 cells rising from 1.6% to 2.2%. By staining the PBMC with anti-CD8, tetramer and Annexin V, we determined that the clonal cells were undergoing rapid cell death, with over 90% of tetramer-positive cells dying within 48 hours.

In mice, the adoptive transfer of CTL is effective in controlling infection with influenza and LCMV, consistent with the survival and expansion of the infused cells (Baenziger, et al. 1986, Lukacher, et al. 1984, Yap, et al. 1978). The frequent pre-treatment of mice with irradiation apparently facilitates expansion of these cells, suggesting that removal of lymphocytes provides a stimulus for re-population of lymphoid tissue (Bell and Shand 1975). In this regard, it may be significant that successful adoptive transfer in humans has been limited so far to BMT recipients,

who would have received prior chemo- or irradiation therapy. Moreover, cell transfer in BMT recipients are performed in a setting of an actively regenerating immune system, which is not the case in AIDS patients or recipients of solid organ transplants. In addition, the lymphocytes which are transferred in murine experiments are often resting spleen cells; in contrast, we used antigen-stimulated, cultured CTL which may possess different characteristics *in vivo* due to the non-physiological expansion process. The development of the mature, stimulated phenotype seen in our clones, characterized by upregulation of Fas and absence of CD28 expression, may be a result of overstimulation of HIV-specific lymphocytes both *in vivo* (prior to culture) and *in vitro*. The rapid and prolonged expansion which these cells undergo may render them susceptible to premature apoptosis, particularly in the absence of sufficient IL-2. The rapid turnover of HIV-specific CTL *in vivo* was confirmed by our observation that prior to transfer, 29% of the Gag-specific cells were undergoing cell death. The infusion of roughly 30% more cells did not alter the anti-viral response; instead, a higher percentage of tetramer-positive cells underwent apoptosis following infusion, implying that a cascade of antigen-specific cell death was triggered. Follow-up of the patient forty days after cell-transfer indicated that the number of Gag-specific CTL had not returned to pre-treatment levels (data not shown) and suggests that the lost CTL were not replenished by expansion of naive cells.

Interestingly, in a previous trial of adoptive transfer, in which a single Nef-specific clone was infused to a patient with AIDS, the clone appeared to drive the selection of viruses containing mutations in the targeted Nef epitope. The selection of escape mutants in this setting implies that the infused CTL survived *in vivo* and exerted pressure on HIV-infected cells. However, in that trial, some of the cell infusions were accompanied by very large doses of IL-2, raising the possibility that the survival of CTL *in vivo* is contingent on CD4⁺ T-cell help, predominantly through IL-2: this may be absent or dysfunctional in HIV-infected patients. The suggestion that CTL require proper CD4⁺ cell function for optimal activity is supported by data in mice and humans. In mice with a null mutation of CD4, infection by lymphocytic

choriomeningitis virus (LCMV) leads to rapid disappearance of anti-viral CTL and poor control of viral replication (Battegay, et al. 1994). Similarly, in immunosuppressed patients whose anti-CMV cellular immunity was restored by adoptive transfer, CTL activity correlated with the level of CMV-specific CD4+ T-cells (Riddell and Greenberg 1995, Walter, et al. 1995). This hypothesis is also supported by recent data which show that HIV-1-specific proliferative responses to p24 were inversely related to viral load (Rosenberg, et al. 1997).

The control of lymphocyte lifespan is thought to be dependent upon the regulated expression of the TNF-receptor family molecule, Fas, which induces a signal cascade leading to cellular apoptosis upon activation by Fas ligand (Lynch, et al. 1995). We and others have shown that SIV and HIV-infected cells upregulate FasL enabling them to reverse-kill antigen-specific CTL in a Fas-dependent manner (Xu, et al. 1997). We now confirm this observation using human cells *in vitro* by demonstrating that a Fas-ligand-expressing cell-line can kill HIV-specific CTL clones in a Fas-dependent manner. It is unclear whether the EBV and CMV-specific CTL clones previously used in BMT patients were expressing Fas, although this would be expected given the *in vitro* expansion protocols. In that case, it would suggest that EBV or CMV-infected cells do not upregulate FasL in the same manner as HIV or SIV. However, it may be worthwhile to investigate this mechanism further for trials of adoptive therapy in other situations, including malignant disease, where Fas ligand is frequently upregulated.

Because human trials of adoptive cell transfer in HIV are laborious and infrequent, the generality of these finding will need to be confirmed by different groups. The finding that HIV-specific CTL undergo apoptosis following adoptive transfer will need to be confirmed. Ideally, controls would be added to include adoptive transfer of HLA-mismatched CTL or adoptive transfer of CTL to healthy controls. However, at the moment these experiments could not be easily conducted. Subsequent trials should take into account the vulnerability of cells cultured *in vitro* to apoptosis and employ means for quantifying their survival *in vivo*. These data

also suggest co-infusion of IL-2 may increase the survival of CTL. Engineering apoptosis-resistant antigen-specific CTL may circumvent the obstacle, but the success of this strategy depends upon better elucidating the mechanisms of cell death *in vivo*.

Summary Of Adoptive Transfer Experiments

In summary, we have conducted three human adoptive transfer experiments:

1. Adoptive transfer of syngeneic lymphocytes from an HIV-uninfected individual to his HIV-infected identical twin. We have shown that:
 - i) The TCR repertoire of CD8+ cells is grossly disturbed in AIDS.
 - ii) CD8+ V-beta expansions may occur early or late following adoptive transfer of lymphocytes.
 - iii) Expansions of CD4 and CD8 cells occur *in vivo* and are maximal at around 6 weeks following transfer.
2. Adoptive transfer of autologous HIV-specific CTL to an HIV-infected patient with a low CD4 count. We have shown that:
 - i) Adoptive transfer of autologous HIV-specific CTL is feasible and safe.
 - ii) No changes to virus load occur following CTL transfer.
 - iii) Restimulated HIV-specific CTL responses are cyclical in nature.
 - iv) Expanded CTL express Fas and are susceptible to Fas-mediated apoptosis.
 - v) IL-2 protects cultured CTL from Fas-induced apoptosis.
3. Adoptive transfer of autologous HIV-specific CTL to an HIV-infected patient with a moderate CD4 count. We have shown that:
 - i) Despite a moderate CD4 count and a high virus load, transfer of HIV-specific CTL does not reduce viral burden.
 - ii) HIV-specific CTL rapidly undergo apoptosis *in vivo* following adoptive transfer.

- iii) HIV-specific CTL can be triggered to undergo Fas-mediated apoptosis using a Fas ligand expressing cell line.

Adoptive transfer of CTL for the therapy of AIDS has not yet proven useful and does not justify the intensive labour required. A better understanding of the mechanisms of *in vivo* lymphocyte repopulation is needed to circumvent the problem of cell survival. In particular, it will be necessary to study more closely animal models of lymphocyte repopulation with particular regard to the factors which mediate peripheral positive selection of lymphocytes and lymphocyte expansion. The phenotype of the adoptively transferred cell will also require study to determine if memory CTL are capable of longterm expansion. These *in vivo* factors have important implications not only for HIV and AIDS but for the adoptive cell therapy of tumours and other infectious diseases where prior ablation or suppression of lymphoid cells is not a pre-condition. In addition, by identifying the mechanisms which trigger lymphocyte death, we may be able to genetically modify cells to resist apoptosis. For instance, some investigators have attempted to transfect virus-specific CTL with a chimeric protein vector expressing the cytoplasmic domain of IL-2 with the extracellular portion of the GM-CSF receptor – the advantage of this approach is the option of infusing GM-CSF to patients in order to stimulate IL-2 secretion from CTL and hopefully obviate the need for functional CD4 helper cells. Other groups have transfected T cells with “universal T cell receptors” which couple the extracellular domain of CD4 or other HIV recognizing domains with the TCR cytoplasmic domain in an attempt to bypass MHC restriction (Yang, et al. 1997). In this latter approach, the therapeutic efficacy of these cells will likely also depend on the *in vivo* availability of lymphocyte growth factors.

Despite its limitations for AIDS, CTL therapy may prove useful in some other settings including the control of Herpes group viruses other than EBV and CMV. For instance, HHV-8 is a gammaherpesvirus which share with EBV the propensity to cause tumours in immunosuppressed individuals. These tumours include Kaposi's sarcoma and body cavity lymphomas. Cultivation of HHV-8 specific CTL *in vitro*

may offer an alternative therapy for these diseases since these diseases are normally controlled by the intact immune system.

REFERENCES

Aebischer, T., Moskophidis, D., Rohrer, U. H., Zinkernagel, R. M. and Hengartner, H. (1991). In vitro selection of lymphocytic chorio-meningitis virus escape mutants by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. (USA)*. 88, 11047-11051.

Ahn, K., Angulo, A., Ghazal, P., Peterson, P. A., Yang, Y. and Fruh, K. (1996). Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc Natl Acad Sci U S A* 93, 10990-5.

Alexander Miller, M. A., Leggatt, G. R. and Berzofsky, J. A. (1996). Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc-Natl-Acad-Sci-U-S-A* 93, 4102-7.

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. and Berger, E. A. (1996). CC CKR5 : a RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272, 1955-1958.

Ariyoshi, K., Harwood, E., Chiengsong-Popov, R. and Weber, J. (1992). Is clearance of HIV-1 viraemia at seroconversion mediated by neutralising antibodies ? *Lancet* 340, 1257-8.

Baenziger, J., Hengartner, H., Zinkernagel, R. M. and Cole, G. A. (1986). Induction or prevention of immunopathological disease by cloned cytotoxic T cell lines specific for lymphocytic choriomeningitis virus. *Eur J Immunol* 16, 387-93.

Baier, M., Werner, A., Bannert, N., Metzner, K. and Kurth, R. (1995). HIV suppression by interleukin-16. *Nature* 378,

Barker, E., Mackewicz, C. E. and Levy, J. A. (1995). Effects of Th1 and Th2 cytokines on CD8+ cell response against human immunodeficiency virus: Implications for long-term survival. *Proc Natl Acad Sci* 92, 11135-11139.

Battegay, M., Moskophidis, D., Rahemtulla, A., Hengartner, H., Mak, T. W. and Zinkernagel, R. M. (1994). Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. *J Virol* 68, 4700-4.

Bell, E. B. (1979). Antigen-laden cells in thoracic duct lymph. Implications for adoptive transfer experiments. *Immunology* 38, 797-808.

Bell, E. B. and Shand, F. L. (1975). Changes in lymphocyte recirculation and liberation of the adoptive memory response from cellular regulation in irradiated recipients. *Eur J Immunol* 5, 1-7.

Bell, E. B., Sparshott, S. M., Drayson, M. T. and Ford, W. L. (1987). The stable and permanent expansion of functional T lymphocytes in athymic nude rats after a single injection of mature T cells. *J Immunol* 139, 1379-1385.

Beral, V., Peterman, T., Berkelman, R. and Jaffe, H. (1991). AIDS-associated non-Hodgkin lymphoma. *Lancet* 337, 805-809.

Bex, F., Hermans, P., Sprecher, S., Achour, A., Badjou, R., Desgranges, C., Cogniaux, J., Franchioli, P., Vanhulle, C., Lachgar, A. and et, a. l. (1994). Syngeneic adoptive transfer of anti-human immunodeficiency virus (HIV-1)-primed lymphocytes from a vaccinated HIV-seronegative individual to his HIV-1-infected identical twin. *Blood* 84, 3317-26.

Bhardwaj, N., Bender, A., Gonzalez, N., Bui, L. K., Garrett, M. C. and Steinman, R. M. (1994). Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells. *J Clin Invest* 94, 797-807.

Biddison, W. E., Sharrow, S. O. and Shearer, G. M. (1981). T cell subpopulations required for the human cytotoxic T lymphocyte response to influenza virus: evidence for T cell help. *J-Immunol* 127, 487-91 issn: 0022-1767.

Biti, R., FFrench, R., Young, J., Bennetts, B. and Stewart, G. (1997). HIV-1 infection in an individual homozygous for the CCR5 deletion allele. *Nature Medicine* 3, 252-3.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. and Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329, 506-511.

Blackburn, E. H. (1991). Structure and function of telomeres. *Nature* 350, 569-73.

Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J. and Springer, T. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 382, 829-833.

Boldt Houle, D. M., Jamieson, B. D., Aldrovandi, G. M., Rinaldo, C. R., Jr., Ehrlich, G. D. and Zack, J. A. (1997). Loss of T cell receptor Vbeta repertoires in HIV type 1-infected SCID-hu mice. *AIDS-Res-Hum-Retroviruses* 13, 125-34.

Bonneau, R. H. and Jennings, S. R. (1990). Herpes simplex virus-specific cytolytic T lymphocytes restricted to a normally low responder H-2 allele are protective in vivo. *Virology* 174, 599-604 issn: 0042-6822.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M. and Oldstone, M. B. (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of

viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 68, 6103-10.

Borrow, P., Lewicki, H., Horwitz, M. S., Pfeffer, N., Myers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Olstone, M. B. A. and Shaw, G. M. (1996). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Med* 3, 205-211.

Borthwick, N. J., Bofill, M., Gombert, W., Akbar, A. N., Medina, E., Sagawa, K., Lipman, M. C., Johnson, M. A. and Janossy, G. (1994). Lymphocyte activation in HIV-1 infection II : functional defects of CD28- T-cells. *AIDS* 8, 431-441.

Boudet, F., Lecoecur, H. and Gougeon, M.-L. (1996). Apoptosis associated with ex vivo down-regulation of Bcl-2 and up-regulation of Fas in potential cytotoxic CD8+ T lymphocytes during HIV infection. *J. Immunol.* 156, 2282-2293.

Boyer, V., Smith, L. R., Ferre, F., Pezzoli, P., Trauger, R. J., Jensen, F. C. and Carlo, D. J. (1993). T cell receptor V beta repertoire in HIV-infection individuals: lack of evidence for selective V beta deletion. *Clin-Exp-Immunol* 92, 437-41.

Braciale, T. J., Andrew, M. E. and Braciale, V. L. (1981). Simultaneous expression of H2-restricted and alloreactive recognition by a cloned line of influenza virus-specific CTL. *J. Exp. Med.* 153, 1371-1376.

Brinchmann, J. E., Gaudernack, G. and Vartdal, F. (1990). CD8+ T cells inhibit HIV replication in naturally infected CD4+ T cells. Evidence for a soluble inhibitor. *J. Immunol.* 144, 2961-2966.

Brugnoni, D., Prati, E., Malacarne, F., Gorla, R., Airo, P. and Cattaneo, R. (1996). The primary response to HIV infection is characterised by an expansion of activated CD8+ CD28- cells. *AIDS 10*, 104-106.

Buchmeier, M. J., Welsh, R. M., Dutko, F. J. and Oldstone, M. B. A. (1980). The virology and immunology of lymphocytic choriomeningitis virus infection. *Adv. Immunol. 30*, 275-331.

Bunce, M., O'Neill, C. M., Barnardo, M. C., Krausa, P., Browning, M. J., Morris, P. J. and Welsh, K. I. (1995). Phototyping : comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers. *Tissue Antigens 46*, 355-367.

Burger, H., Weiser, B., Robinson, W., Lifson, J., Engleman, E., Rousioux, C., Brun-Vezinet, F., Barre-Sinoussi, F., Montagnier, L. and Chermann, J.-C. (1985). Transient antibody to LAV/HTLV-III and T-lymphocyte abnormalities in the wife of a man who developed AIDS. *Ann. Int. Med. 103*, 545-547.

Burns, D. P. and Desrosiers, R. C. (1994). Envelope sequence variation, neutralizing antibodies, and primate lentivirus persistence. *Curr Top Microbiol Immunol 188*, 185-219.

Buseyne, F., Janvier, G., Fleury, B., Schmidt, D. and Riviere, Y. (1994). Multispecific and heterogeneous recognition of the gag protein by cytotoxic T lymphocytes (CTL) from HIV-infected patients: factors other than the MHC control the epitopic specificities. *Clin Exp Immunol 97*, 353-60.

Callan, M. F. C., Steven, N., Krausa, P., Wilson, J. D. K., Moss, P. A. H., Gillespie, G. M., Bell, J. I., Rikinson, A. B. and McMichael, A. J. (1996). Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. *Nature Medicine In press.*

Cannon, M. J., Openshaw, P. J. M. and Askonas, B. (1988). Cytotoxic cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J. Exp. Med.* 168, 1163-1168.

Cao, Y., Qin, L., Zhang, L., Safrit, J. and Ho, D. D. (1995). Virological and immunological characterisation of long-term survivors of HIV-1 infection. *New Engl. J. Med.* 332, 201-208.

Cameron, P. U., Freudenthal, P. S., Barker, J. M., Gezelter, S., Inaba, K. and Steinman, R. M. (1992). Dendritic cells exposed to HIV-1 transmit a vigorous cytopathic infection to CD4+ T cells. 257, 383-387.

Carmichael, A., Jin, X., Sissons, P. and Borysiewicz, L. (1993). Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. *J Exp Med* 177, 249-56.

Carr, C. M., Chaudhry, C. and Kim, P. S. (1997). Influenza hemagglutinin is spring-loaded by a metastable native conformation [In Process Citation]. *Proc Natl Acad Sci U S A* 94, 14306-13.

Carroll, R. G., Riley, J. L., Levine, B., Feng, Y., Kaushal, S., Ritchey, D. W., Bernstein, W., Weislow, O. S., Brown, C. R., Berger, E. A., June, C. H. and St. Louis, D. C. (1997). Differential regulation of HIV-1 fusion cofactor expression by CD28 costimulation of CD4+ T-cells. *Science* 276, 273-279.

Celada, F. (1967). Quantitative studies of the adoptive immunological memory in mice. II. Linear transmission of cellular memory. *J Exp Med* 125, 199-211.

Chan, A. C., Iwashima, M., Turck, C. W. and Weiss, A. (1992). ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 71, 649-62.

Chen, Z. W., Kou, Z. C., Lekutis, C., Shen, L., Zhou, D., Halloran, M., Li, J., Sodroski, J., Lee Parritz, D. and Letvin, N. L. (1995). T cell receptor V beta repertoire in an acute infection of rhesus monkeys with simian immunodeficiency viruses and a chimeric simian-human immunodeficiency virus. *J-Exp-Med* 182, 21-31.

Chen, Z. W., Shen, L., Regan, J. D., Kou, Z., Ghim, S. H. and Letvin, N. L. (1996). The T cell receptor gene usage by simian immunodeficiency virus gag-specific cytotoxic T lymphocytes in rhesus monkeys. *J-Immunol* 156, 1469-75.

Cheynier, R., Henrichwark, S., Hadida, F., Pelletier, E., Oksenhendler, E., Autran, B. and Wain Hobson, S. (1994). HIV and T cell expansion in splenic white pulps is accompanied by infiltration of HIV-specific cytotoxic T lymphocytes. *Cell* 78, 373-87.

Chia, W. K., Freedman, J., Li, X., Salit, I., Kardish, M. and Read, S. E. (1995). Programmed cell death induced by HIV type 1 antigen stimulation is associated with a decrease in cytotoxic T lymphocyte activity in advanced HIV type 1 infection. *Aids Research and Human Retroviruses* 11, 249-256.

Chien, Y. H., Jores, R. and Crowley, M. P. (1996). Recognition by gamma/delta T cells. *Annu Rev Immunol* 14, 511-32.

Choe, H., Farzan, M., Sun, Y., sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. and Sodroski, J. (1996). The β -chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85, 1135-1148.

Chun, T. W., Carruth, L., Finzi, D., Shen, X., DiGiuseppe, J. A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T. C., Kuo, Y. H.,

Brookmeyer, R., Zeiger, M. A., Barditch-Crovo, P. and Siliciano, R. F. (1997). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection . *Nature* 387, 183-8.

Chun, T. W., Finzi, D., Margolick, J., Chadwick, K., Schwartz, D. and Siliciano, R. F. (1995). In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med* 1, 1284-90.

Clerici, M., Hakim, F., Venzon, D., Blatt, S., Hendrix, C., Wynn, T. and Shearer, G. M. (1993). Changes in IL-2 and IL-4 production in asymptomatic HIV-seropositive individuals. *J. Clin. Invest.* 91, 759.

Clerici, M., Sarin, A., Coffman, R. L., Wynn, T. A., Blatt, S. P., Hendrix, C. W., Wolf, S. F., Shearer, G. M. and Henkart, P. A. (1994). Type 1/type 2 cytokine modulation of T-cell programmed cell death as a model for human immunodeficiency virus pathogenesis. *Proc-Natl-Acad-Sci-U-S-A* 91, 11811-5.

Clerici, M. and Shearer, G. (1993). A Th1-> Th2 switch is a critical step in the aetiology of HIV infection. *Immunol. Today* 14, 107-111.

Clerici, M. and Shearer, G. (1994). The Th1-Th2 hypothesis of HIV infection : new insights. *Immunology Today* 15, 575-581.

Clerici, M., Wynn, T. A., Berzofsky, J. A., Blatt, S. P., Hendrix, C. W., Sher, A., Coffman, R. L. and Shearer, G. M. (1994). Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus. *J-Clin-Invest* 93, 768-75.

Cocchi, F., DeVico, A. L., Garzino, D. A., Arya, S. K., Gallo, R. C. and Lusso, P. (1995). Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* 270, 1811-5.

Coffin, J. M. (1995). HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* 267, 483-9.

Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D. and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* 391, 397-401.

Colonna, M. (1997). Specificity and function of immunoglobulin superfamily NK cell inhibitory and stimulatory receptors. *Immunol Rev* 155, 127-33.

Colonna, M. and Samaridis, J. (1995). Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells . *Science* 268, 405-8.

Connors, M., Kovacs, J. A., Krevat, S., Gea-Banaloche, J. C., Sneller, M. C., Flanigan, M., Metcalf, J. A., Walker, R. E., Falloon, J., Baseler, M., Stevens, R., Feuerstein, I., Masur, H. and Lane, H. C. (1997). HIV infection induces changes in CD4+ t-cell phenotype and depletions within the CD4+ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. *Nature Med* 3, 533-540.

Contu, L., La Nasa, G., Arras, M., Pizzati, A., Vacca, A., Carcassi, C., Ledda, A., Boero, R., Orru, S., Pintus, A. and et al. (1993). Allogeneic bone marrow transplantation combined with multiple anti-HIV-1 treatment in a case of AIDS. *Bone-Marrow-Transplant* 12, 669-71.

Cooper, D., Gold, J., Maclean, P., Donovan, B., Finlayson, R., Barnes, T., Michelmore, H., Brooke, P. and Penny, R. (1985). Acute AIDS retrovirus infection. Definition of a clinical illness associated with seroconversion. *Lancet* i, 537-540.

Craven, R. C., Bennett, R. P. and Wills, J. W. (1991). Role of the avian retroviral protease in the activation of reverse transcriptase during virion assembly. *J Virol* 65, 6205-17.

Culmann, B., Gomard, E. and Kieny, M.-P. (1991). Six epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 nef protein. *J. Immunol.* 146, 1560-1565.

Culmann, B., Gomard, E., Kieny, M.-P., Guy, B., Dreyfus, F., Saimot, A.-G., Sereni, D. and Levy, J.-P. (1989). An antigenic peptide of HIV-1 NEF protein recognized by cytotoxic T lymphocytes of seropositive individuals in association with different HLA-B molecules. *Eur. J. Immunol.* 19, 2383-2386.

Davis, K. C., Hayward, A., Ozturk, G. and Kohler, P. F. (1983). Lymphocyte transfusion in case of acquired immunodeficiency syndrome [letter]. *Lancet* 1, 599-600.

de Campos-Lima, P., Torsteinsdottir, S., Cuomo, L., Klein, G., Sulitzeanu, D. and Masucci, M. G. (1993). Antigen processing and presentation by EBV-carrying cell lines: cell-phenotype dependence and influence of the EBV-encoded LMP1. *Int J Cancer* 53, 856-62.

de Campos-Lima, P. O., Levitsky, V., Lee, S. P., Hu, L. F., Rickinson, A. B. and Masucci, M. G. (1994). T cell responses and virus evolution : Loss of HLA A11-restricted CTL epitopes in EBV isolates from highly A11-positive populations by selective mutation of anchor residues. *J. Exp. Med.* 179, 1297-1305.

De Maria, A., Pantaleo, G., Schnittman, S. M., Greenhouse, J. J., Baseler, M., Orenstein, J. M. and Fauci, A. S. (1991). Infection of CD8+ T lymphocytes with HIV. Requirement for interaction with infected CD4+ cells and induction of infectious virus from chronically infected CD8+ cells. *J Immunol* 146, 2220-6.

de Vries, J. E. and Spits, H. (1984). Cloned human cytotoxic T lymphocyte (CTL) lines reactive with autologous melanoma cells. I. In vitro generation, isolation, and analysis to phenotype and specificity. *J-Immunol* 132, 510-9 issn: 0022-1767.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. and Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381, 661-666.

Dong, T., Boyd, D., Rosenberg, W., Alp, N., Takiguchi, M., McMichael, A. J. and Rowland-Jones, S. L. (1996). Increased affinity for HLA-B35 of an influenza A epitope modified at an "anchor" residue results in an antagonist peptide. *Eur. J. Immunol.* 26, 335-339.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G. and Doms, R. W. (1996). A dual-tropic primary HIV-1 isolate that uses Fusin and the β -chemokine receptors CKR-5, CKR-3 and CKR-2b as fusion cofactors. *Cell* 85, 1149-1158.

Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. and Paxton, W. A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381, 667-673.

Effros, R. B., Allsopp, R., Chiu, C.-P., Hausner, M. A., Hirji, K., Wang, L., Harley, C. B., Villeponteau, B., West, M. D. and Giorgi, J. V. (1996). Shortened telomeres in the expanded CD28- CD8+ subset in HIV disease implicate replicative senescence in HIV pathogenesis. *AIDS* 10, F17-22.

Emilie, D., Maillot, M. C., Nicolas, J. F., Fior, R. and Galanaud, P. (1992).

Antagonistic effect of interferon-gamma on tat-induced transactivation of HIV long terminal repeat. *J-Biol-Chem* 267, 20565-70.

Estaquier, J., Idziorek, T., Zou, W., Emilie, D., Farber, C. M., Bourez, J. M. and Ameisen, J. C. (1995). T helper type 1/T helper type 2 cytokines and T cell death: preventive effect of interleukin 12 on activation-induced and CD95 (FAS/APO-1)-mediated apoptosis of CD4+ T cells from human immunodeficiency virus-infected persons. *J-Exp-Med* 182, 1759-67.

Ewing, C., Allan, W., Daly, K., Hou, S., Cole, G. A., Doherty, P. C. and Blackman, M. A. (1994). Virus-specific CD8+ T-cell responses in mice transgenic for a T-cell receptor beta chain selected at random. *J-Virol* 68, 3065-70 issn: 0022-538x.

Faith, A., Yssel, H., O'Hehir, R. E. and Lamb, J. R. (1996). Reversal of the inhibitory effects of HIV-gp120 on CD4+ T cells by stimulation through the CD28 pathway. *Clin Exp Immunol* 105, 225-30.

Farzadegan, H., Polis, M. A., Wolinsky, S. M., Rinaldo, C. R., Jr., Sninsky, J. J., Kwok, S., Griffith, R. L., Kaslow, R. A., Phair, J. P., Polk, B. F. and et al. (1988). Loss of human immunodeficiency virus type 1 (HIV-1) antibodies with evidence of viral infection in asymptomatic homosexual men. A report from the Multicenter AIDS Cohort Study . *Ann Intern Med* 108, 785-90.

Fauci, A. S. (1993). immunopathogenesis of HIV infection. *J.AIDS* 6, 655-662.

Fauci, A. S., Pantaleo, G., Stanley, S. and Weissman, D. (1996). Immunopathogenic mechanisms of HIV infection. *Ann-Intern-Med* 124, 654-63.

Fayolle, C., Deriaud, E. and Leclerc, C. (1991). In vivo induction of cytotoxic T cell response by a free synthetic peptide requires CD4+ T cell help. *J Immunol* 147, 4069-73.

Feng, Y., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996). HIV-1 entry cofactor : functional cDNA cloning of a seven-transmembrane G protein-coupled receptor. *Science* 272, 872-877.

Fisher, B., Packard, B. S., Read, E. J., Carrasquillo, J. A., Carter, C. S., Topalian, S. L., Yang, J. C., Yolles, P., Larson, S. M. and Rosenberg, S. A. (1989). Tumor localization of adoptively transferred indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma. *J Clin Oncol* 7, 250-61.

Fricker, J. (1996). Baboon xenotransplant fails but patient improves. *Lancet* 347, 457.

Fruh, K., Ahn, K., Djaballah, H., Sempe, P., van Endert, P. M., Tampe, R., Peterson, P. A. and Yang, Y. (1995). A viral inhibitor of peptide transporters for antigen presentation. *Nature* 375, 415-8.

Gao, X. M., Zheng, B., Liew, F. Y., Brett, S. and Tite, J. (1991). Priming of influenza virus-specific cytotoxic T lymphocytes vivo by short synthetic peptides. *J Immunol* 147, 3268-73.

Garboczi, D. N., Ghosh, P., Utz, U., Fan, Q. R., Biddison, W. E. and Wiley, D. C. (1996). Structure of the complex between human T-cell receptor, viral peptide and HLA-A2 [comment]. *Nature* 384, 134-41.

Garcia, K. C., Degano, M., Stanfield, R. L., Brunmark, A., Jackson, M. R., Peterson, P. A., Teyton, L. and Wilson, I. A. (1996). An alphabeta T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. *Science* 274, 209-19.

- Garrett, T. P. J., Saper, M. A., Bjorkman, P. J., Strominger, J. L. and Wiley, D. C. (1989). Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature* 342, 692.
- Gaynor, R. B. (1995). Regulation of HIV-1 gene expression by the transactivator protein Tat. *Curr Top Microbiol Immunol* 193, 51-77.
- Gehri, R., Hahn, S., Rothen, M., Steuerwald, M., Nuesch, R. and Erb, P. (1996). The Fas receptor in HIV infection: Expression on peripheral blood lymphocytes and role in the depletion of T cells. *Aids (Philadelphia)* 10, 9-16.
- Germain, R. N. (1991). Antigen presentation: The second class story. *Nature* 353, 605-606.
- Gilbert, M. J., Riddell, S. R., Plachter, B. and Greenberg, P. D. (1996). Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature* 383, 720-2.
- Giri, N., Vowels, M. R. and Ziegler, J. B. (1992). Failure of allogeneic bone marrow transplantation to benefit HIV infection. *J Paediatr Child Health* 28, 331-333.
- Goldberg, A. L. and Rock, K. L. (1992). Proteolysis, proteasomes and antigen presentation. 357, 375-378.
- Gorski, J., Piatek, T., Yassai, M., Gorski, J. and Maslanka, K. (1995). Improvements in repertoire analysis by CDR3 size spectratyping. Bifamily PCR. *Ann N Y Acad Sci* 756, 99-102.

Gotch, F. M., Nixon, D. F., Alp, N., McMichael, A. J. and Borysiewicz, L. K. (1990). High frequency of memory and effector gag specific cytotoxic T lymphocytes in HIV seropositive individuals. *Int. Immunol.* 2, 707.

Goulder, P. J., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A. J. and Rowland-Jones, S. (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 3, 212-7.

Grant, M. D., Smaill, F. M., Singal, D. P. and Rosenthal, K. L. (1992). The influence of lymphocyte counts and disease progression on circulating and inducible anti-HIV-1 cytotoxic T-cell activity in HIV-1-infected subjects. *Aids* 6, 1085-94.

Graziosi, C., Pantaleo, G., Butini, L., Demarest, J. F., Saag, M. S., Shaw, G. M. and Fauci, A. S. (1993). Kinetics of human immunodeficiency virus type 1 (HIV-1) DNA and RNA synthesis during primary HIV-1 infection. *Proc Natl Acad Sci U S A* 90, 6405-9.

Graziosi, C., Pantaleo, G., Gantt, K. R., Fortin, J.-P., Demarest, J. F., Cohen, O. J., Sekaly, R. P. and Fauci, A. S. (1994). Lack of evidence for the dichotomy of Th1 and Th2 predominance in HIV-infected individuals. *Science* 265, 248-252.

Greenberg, P. D. (1991). Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv Immunol* 49, 281-355.

Grimm, E. A., Mazumder, A., Zhang, H. Z. and Rosenberg, S. A. (1982). Lymphokine-activated killer cell phenomenon: lysis of natural killer resistant fresh solid tumor cells by interleukin-2 activated autologous human peripheral blood lymphocytes. *J. Exp. Med.* 155, 1823-1841.

Gupta, K. K. (1993). Acute immunosuppression with HIV seroconversion. *New Engl. J. Med.* 328, 228-9.

Hadida, F., Haas, G., Zimmerman, N., Hosmalin, A., Spohn, R., Samri, A., Jung, G., Debre, P. and Autran, B. (1995). CTLs from lymphoid organs recognise an optimal HLA-A2-restricted and HLA-B52-restricted nonapeptide and several epitopes in the C-terminal region of HIV-1 nef. *J. Immunol.* 154, 4174-4186.

Haque, T., Amlot, P. L., Helling, N., Thomas, J. A., Sweny, P., Rolles, K., Burroughs, A. K., Prentice, H. G. and Crawford, D. H. (1998). Reconstitution of EBV-specific T cell immunity in solid organ transplant recipients. *J Immunol* 160, 6204-9.

Harrer, E., Harrer, T., Barbosa, P., Feinberg, M., Johnson, R. P., Buchbinder, S. and Walker, B. D. (1996). Recognition of the highly conserved YMDD region in the human immunodeficiency virus type 1 reverse transcriptase by HLA-A2-restricted cytotoxic T lymphocytes from an asymptomatic long-term nonprogressor. *J Infect Dis* 173, 476-9.

Harrer, T., Harrer, E., Kalams, S., Barbosa, P., Trocha, A., Johnson, R. P., Elbeik, T., Feinberg, M. B., Buchbinder, S. P. and Walker, B. D. (1996). Cytotoxic T lymphocytes in asymptomatic long-term non-progressing HIV-1 infection. *J. Immunol.* 156, 2616-2623.

Harrer, T., Harrer, E., Kalams, S., Trocha, A., Johnson, R. P., Elbeik, T., Feinberg, M. B., Cao, H., Ho, D. D., Buchbinder, S. P. and Walker, B. D. (1996). Strong cytotoxic T cells and weak neutralising antibodies in long-term non-progressing HIV-1 infection. *AIDS Res. Hum. Retroviruses*

Hassett, J. M., Zaroulis, C. G., Greenberg, M. L. and Siegal, F. P. (1983). Bone marrow transplantation in AIDS [letter]. *N-Engl-J-Med* 309, 665.

Hawes, G. E., Struyk, L. and van den Elsen, P. J. (1993). Differential usage of T cell receptor V gene segments in CD4+ and CD8+ subsets of T lymphocytes in monozygotic twins. *J Immunol* 150, 2033-2045.

Hedrick, S. M., Cohen, D. I., Nielsen, E. A. and Davis, M. M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* 308, 149-53.

Henkart, P. A. (1994). Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity* 1, 343-6.

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H. and Johnson, D. (1995). Herpes simplex virus turns off the TAP to evade host immunity. *Nature* 375, 411-5.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M. and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 373, 123-6.

Ho, M., Armstrong, J., McMahon, D., Pazin, G., Huang, X. L., Rinaldo, C., Whiteside, T., Tripoli, C., Levine, G., Moody, D. and et al. (1993). A phase 1 study of adoptive transfer of autologous CD8+ T lymphocytes in patients with acquired immunodeficiency syndrome (AIDS)-related complex or AIDS. *Blood* 81, 2093-101.

Holland, H. K., Saral, R., Rossi, J. J., Donnenberg, A. D., Burns, W. H., Beschoner, W. E., Farzadegan, H., Jones, R. J., Quinnan, G. V., Vogelsang, G. B. and et al. (1989). Allogeneic bone marrow transplantation, zidovudine, and human immunodeficiency virus type 1 (HIV-1) infection. Studies in a patient with non-Hodgkin lymphoma. *Ann-Intern-Med* 111, 973-81.

Holmes, E. C., Zhang, L. Q., Simmonds, P., Ludlam, C. A. and Leigh-Brown, A. J. (1992). Convergent and divergent sequence evolution in the surface envelope glycoprotein of human immunodeficiency virus type I within a single infected patient. *PNAS In Press*,

Hou, S., Mo, X. Y., Hyland, L. and Doherty, P. C. (1995). Host response to Sendai virus in mice lacking class II major histocompatibility complex glycoproteins. *J-Virol* 69, 1429-34.

Hurme, M., Hetherington, C. M., Chandler, P. R. and Simpson, E. (1978). Cytotoxic T-cell responses to H-Y: mapping of the Ir genes. *J-Exp-Med* 147, 758-67 issn: 0022-1007.

Imberti, L., Sottini, A., Bettinardi, A., Puoti, M. and Primi, D. (1991). Selective depletion in HIV infection of T cells that bear specific T cell receptor V beta sequences. *Science* 254, 860-2.

Islam, D., Wretling, B., Lindberg, A. A. and Christensson, B. (1996). Changes in the peripheral blood T-Cell receptor V beta repertoire in vivo and in vitro during shigellosis. *Infect-Immun* 64, 1391-9.

Israel Biet, D., Labrousse, F., Tourani, J. M., Sors, H., Andrieu, J. M. and Even, P. (1992). Elevation of IgE in HIV-infected subjects: a marker of poor prognosis. *J-Allergy-Clin-Immunol* 89, 68-75.

Itoh, K., Tilden, A. B. and Balch, C. M. (1986). Interleukin 2 activation of cytotoxic T-lymphocytes infiltrating into human metastatic melanomas. *Cancer Res* 46, 3011-7.

Jabbar, M. A. (1995). The human immunodeficiency virus type 1 Vpu protein: roles in virus release and CD4 downregulation. *Curr Top Microbiol Immunol* 193, 107-20.

Jacobson, M. A., Zegans, M., Pavan, P. R., O'Donnel, J., Sattler, F., Rao, N., Owens, S. and Pollard, R. (1997). Cytomegalovirus retinitis after initiation of highly active antiretroviral therapy. *Lancet* 349, 1443-1445.

Jacobson, S., Shida, H., McFarlin, D. E., Fauci, A. and Koenig, S. (1990). Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-1 pX in patients with HTLV-1 associated neurological disease. *348*, 245-248.

Jassey, C., Johnson, R. P., Navia, B. A., Worth, J. and Walker, B. D. (1992). Detection of a vigorous HIV-1-specific cytotoxic T lymphocyte response in cerebrospinal fluid from infected persons with AIDS dementia complex. *J Immunol* 149, 3113-9.

Jones, T. R., Wiertz, E. J., Sun, L., Fish, K. N., Nelson, J. A. and Ploegh, H. L. (1996). Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc Natl Acad Sci U S A* 93, 11327-33.

Kagi, D., Seiler, P., Pavlovic, J., Ledermann, B., Burki, K., Zinkernagel, R. M. and Hengartner, H. (1995). The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. *Eur-J-Immunol* 25, 3256-62.

Kalams, S. A. and Walker, B. D. (1995). Cytotoxic T lymphocytes and HIV-1 related neurologic disorders. *Curr Top Microbiol Immunol* 202, 79-88.

Karre, K., Ljunggren, H. G., Piontek, G. and Kiessling, R. (1986). Selective rejection of H-2 deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319, 675.

Katz, R. A. and Skalka, A. M. (1994). The retroviral enzymes. *Annu Rev Biochem* 63, 133-73.

Kerkau, T., Schmitt, L. R., Schimpl, A. and Wecker, E. (1989). Downregulation of HLA class I antigens in HIV-1-infected cells. *Aids Res Hum Retroviruses* 5, 613-20.

Klein, M. R., Van, B. C. A., Holwerda, A. M., Garde, S. R. K., Bende, R. J., Keet, I. P. M., Eeftinck, S. J. K. M., Osterhaus, A. D. M. E., Schuitemaker, H. and Miedema, F. (1995). Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. *Journal of Experimental Medicine* 181, 1365-1372.

Klenerman, P., Phillips, R. E., Rinaldo, C. R., Wahl, L. M., Ogg, G., May, R. M., McMichael, A. J. and Nowak, M. A. (1996). Cytotoxic T lymphocytes and viral turnover in HIV type 1 infection. *Proc Natl Acad Sci U S A* 93, 15323-8.

Klimas, N., Patarca, R., Walling, J., Garcia, R., Mayer, V., Moody, D., Okarma, T. and Fletcher, M. A. (1994). Clinical and immunological changes in AIDS patients following adoptive therapy with activated autologous CD8 T cells and interleukin-2 infusion. *AIDS* 8, 1073-81.

Koenig, S., Conley, A. J., Brewah, Y. A., Jones, G. M., Leath, S., Boots, L. J., Davey, V., Pantaleo, G., Demarest, J. F., Carter, C., Wannebo, C., Yannelli, J. R., Rosenberg, S. A. and Lane, H. C. (1995). Transfer of HIV-1 specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nature Medicine* 1, 330-336.

Koenig, S., Fuerst, T. R., Wood, L. V., Woods, R. M., Suzich, J. A., Jones, G. M., de la Cruz, V. F., Davey, R. J., Venkatesan, S. and Moss, B. (1990). Mapping the fine

specificity of a cytolytic T cell response to HIV-1 nef protein. *J Immunol* 145, 127-35.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C. and Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68, 4650-5.

Koup, R. A., Sullivan, J. L., Levine, P. H., Brettler, D., Mahr, A., Mazzara, G., McKenzie, S. and Panicali, D. (1989). Detection of major histocompatibility complex class I-restricted, HIV-specific cytotoxic T lymphocytes in the blood of infected hemophiliacs. *Blood* 73, 1909-14.

Kozal, M. J., Shah, N., Shen, N., Yang, R., Fucini, R., Merigan, T. C., Richman, D. D., Morris, D., Hubbell, E., Chee, M. and Gingeras, T. R. (1996). Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. *Nature Medicine* 2, 753-759.

Kradin, R. L., Boyle, L. A., Preffer, F. I., Callahan, R. J., Barlai-Kovach, M., Strauss, H. W., Dubinett, S. and Kurnick, J. T. (1987). Tumor-derived interleukin-2-dependent lymphocytes in adoptive immunotherapy of lung cancer. *Cancer Immunol Immunother* 24, 76-85.

Krown, S. E., Real, F. X., Vadhan Raj, S., Cunningham Rundles, S., Krim, M., Wong, G. and Oettgen, H. F. (1986). Kaposi's sarcoma and the acquired immune deficiency syndrome. Treatment with recombinant interferon alpha and analysis of prognostic factors. *Cancer* 57, 1662-5.

Krown, S. E., Real, F. X., Vadhan Raj, S. and Oettgen, H. F. (1987). Interferon in AIDS. *J-Exp-Pathol* 3, 681-91.

- Lalvani, A., Aidoo, M., Allsopp, C. E., Plebanski, M., Whittle, H. C. and Hill, A. V. (1994). An HLA-based approach to the design of a CTL-inducing vaccine against *Plasmodium falciparum*. *Res-Immunol* 145, 461-8.
- Lamhamedi-Cherradi, S., Culmann-Penciolelli, B., Guy, B., Ly, T. D., Goujard, C., Guillet, J. G. and Gomard, E. (1995). Different patterns of HIV-1-specific cytotoxic T-lymphocyte activity after primary infection. *Aids (Philadelphia)* 9, 421-426.
- Landay, A. L., Mackewicz, C. E. and Levy, J. A. (1993). An activated CD8+ T cell phenotype correlates with anti-HIV activity and asymptomatic clinical status. *Clin. Immunol. Immunopathol.* 69, 106-16.
- Lane, H. C., Masur, H., Longo, D. L., Klein, H. G., Rook, A. H., Quinnan, G. V., Jr., Steis, R. G., Macher, A., Whalen, G., Edgar, L. C. and et al. (1984). Partial immune reconstitution in a patient with the acquired immunodeficiency syndrome. *N-Engl-J-Med* 311, 1099-103.
- Lane, H. C., Zunic, K. M., Wilson, W., Cefali, F., Easter, M., Kovacs, J. A., Masur, H., Leitman, S. F., Klein, H. G., Steis, R. G. and et, a. l. (1990). Syngeneic bone marrow transplantation and adoptive transfer of peripheral blood lymphocytes combined with zidovudine in human immunodeficiency virus (HIV) infection. *Ann Intern Med* 113, 512-9.
- Langlade-Demoyen, P., Michel, F., Hoffenbach, A., Vilmer, E., Dadaglio, G., Garcia-Pons, F., Mayaud, C., Autran, B., Wain-Hobson, S. and Plata, F. (1988). Immune recognition of AIDS virus antigens by human and murine cytotoxic T lymphocytes. *J. Immunol.* 141, 1949-1957.
- Lee, R. S., Grusby, M. J., Glimcher, L. H., Winn, H. J. and Auchincloss, H., Jr. (1994). Indirect recognition by helper cells can induce donor-specific cytotoxic T lymphocytes in vivo. *J-Exp-Med* 179, 865-72.

Levine, B. L., Mosca, J. D., Riley, J. L., Carroll, R. G., Vahey, M. T., Jagodzinski, L. L., Wagner, K. F., Mayers, D. L., Burke, D. S., Weislow, O. S., Louis, D. C. S. and June, C. H. (1996). Antiviral effect and ex vivo CD4+ T cell proliferation in HIV-positive patients as a result of CD28 costimulation. *Science (Washington D C)* 272, 1939-1943.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald Mullen, P. M., Klein, G., Kurilla, M. G. and Masucci, M. G. (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* 375, 685-8.

Levy, J. A. (1993). HIV pathogenesis and long-term survival. *AIDS* 7, 1401-1410.

Levy, J. A., Mackewicz, C. E. and Barker, E. (1996). Controlling HIV pathogenesis: the role of noncytotoxic anti-HIV response of CD8+ T cells. *Immunol. Today* 17, 217-224.

Lieberman, J., Skolnik, P. R., Parkerson, G. R. r., Fabry, J. A., Landry, B., Bethel, J. and Kagan, J. (1997). Safety of autologous, ex vivo-expanded human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte infusion in HIV-infected patients. *Blood* 90, 2196-206.

Lightman, S., Cobbold, S., Waldmann, H. and Askonas, B. A. (1987). Do L3T4+ T cells act as effector cells in protection against influenza virus infection. *Immunology* 62, 139-44.

Lin, Y. L. and Askonas, B. A. (1981). Biological properties of an influenza A virus-specific killer T cell clone. Inhibition of virus replication in vivo and induction of delayed-type hypersensitivity reactions. *J-Exp-Med* 154, 225-34.

Livingstone, W. J., , Moore, M., Innes, D., Bell, J. E., Simmonds, P. and Group), a. t. E. H. T. S. (1996). Frequent infection of peripheral blood CD8+ T-lymphocytes with HIV-1. *Lancet* 348, 649-654.

Loh, E. Y., Elliott, J. F., Cwirla, S., Lanier, L. L. and Davis, M. M. (1989). Polymerase chain reaction with single-sided specificity: analysis of T cell receptor delta chain. *Science* 243, 217-220.

Lohman, B. L., Miller, C. J. and McChesney, M. B. (1995). Antiviral cytotoxic T lymphocytes in vaginal mucosa of Simian immunodeficiency virus-infected rhesus macaques. *Journal of Immunology* 155, 5855-5860.

Loveridge, J., Rosenberg, W. M. C., Kirkwood, B. and Bell, J. I. (1991). The genetic contribution to human T cell receptor repertoire. Submitted

Lucey, D. R., Zajac, R. A., Melcher, G. P., Butzin, C. A. and Boswell, R. N. (1990). Serum IgE levels in 622 persons with human immunodeficiency virus infection: IgE elevation with marked depletion of CD4+ T-cells. *AIDS-Res-Hum-Retroviruses* 6, 427-9.

Lukacher, A. E., Braciale, V. L. and Braciale, T. J. (1984). In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J Exp Med* 160, 814-26.

Luzuriaga, K., Holmes, D., Hereema, A., Wong, J., Panicali, D. L. and Sullivan, J. L. (1995). HIV-1-specific cytotoxic T lymphocyte responses in the first year of life. *J Immunol* 154, 433-43.

Lynch, D. H., Ramsdell, F. and Alderson, M. R. (1995). Fas and FasL in the homeostatic regulation of immune responses. *Immunol-Today* 16, 569-74 issn: 0167-4919.

- Mackewicz, C., Ortega, H. and Levy, J. (1994). Effect of cytokines on HIV replication : lack of identity with the CD8+ antiviral factor. *Cell. Immunol.* 153, 329-343.
- Mackewicz, C. E., Levy, J. A., Cruikshank, W. W., Kornfeld, H. and Center, D. M. (1996). Role of IL-16 in HIV replication. *Nature* 383, 488-9.
- Mackewicz, C. E., Ortega, H. W. and Levy, J. A. (1991). CD8+ cell anti-HIV activity correlates with the clinical state of the infected individual. *J. Clin. Invest.* 87, 1462-1466.
- Mackewicz, C. E., Yang, L. C., Lifson, J. D. and Levy, J. A. (1994). Non-cytolytic CD8 T-cell anti-HIV responses in primary HIV-1 infection. *Lancet* 344, 1671-1674.
- Madden, D. R., Garboczi, D. N. and Wiley, D. C. (1993). The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* 75, 693-708.
- Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A. and Axel, R. (1986). The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 47, 333-48.
- Maggi, E., Giudizi, M. G., Biagiotti, R., Annunziato, F., Manetti, R., Piccinni, M. P., Parronchi, P., Sampognaro, S., Giannarini, L., Zuccati, G. and et al. (1994). Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. *J-Exp-Med* 180, 489-95.
- Maggi, E., Mazzetti, M., Ravina, A., Annunziato, F., de Carli, M., Piccinni, M. P., Manetti, R., Carbonari, M., Pesce, A. M., del Prete, G. and et al. (1994). Ability of

HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells . Science 265, 244-8.

Makela, O. and Mitchison, N. A. (1965). The effect of antigen dosage on the response of adoptively transferred cells. Immunology 8, 549-56.

Makela, O. and Mitchison, N. A. (1965). The role of cell number and source in adoptive immunity. Immunology 8, 539-48.

Matano, T., Shibata, R., Siemon, C., Connors, M., Lane, H. C. and Martin, M. A. (1998). Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J Virol 72, 164-9.

Matloubian, M., Concepcion, R. J. and Ahmed, R. (1994). CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J-Virol 68, 8056-63.

McAdam, S. N., Klenerman, P., Tussey, L., Rowland-Jones, S. L., Lalloo, D., Phillips, R. E., Leigh Brown, A., Gotch, F. M. and McMichael, A. J. (1995). Immunogenic HIV variants that bind to HLA-B8 but fail to stimulate CTL responses. J. Immunol. 155, 2729-2736.

McDougal, J. S., Maddon, P. J., Dalgleish, A. G., Clapham, P. R., Littman, D. R., Godfrey, M., Maddon, D. E., Chess, L., Weiss, R. A. and Axel, R. (1986). The T4 glycoprotein is a cell-surface receptor for the AIDS virus. Cold Spring Harb Symp Quant Biol 51 Pt 2, 703-11.

McKeating, J. A., Gow, J., Goudsmit, J., Pearl, L. H., Mulder, C. and Weiss, R. A. (1989). Characterization of HIV-1 neutralization escape mutants. AIDS 3, 777-784.

- McMichael, A., Gotch, F. and Rothbard, J. (1986). HLA B37 determines an influenza A virus nucleoprotein epitope recognized by cytotoxic T lymphocytes. *J Exp Med* 164, 1397-1406.
- McMichael, A. J., Gotch, F. M., Noble, G. R. and Beare, P. A. S. (1983). Cytotoxic T-cell immunity to influenza. *New Eng.J.Med.* 309, 13-17.
- McMichael, A. J., Gotch, F. M., Santos-Aguado, J. and Strominger, J. L. (1988). Effect of mutations and variations of HLA-A2 on recognition of a virus peptide epitope by cytotoxic T lymphocytes. *Proc Natl Acad Sci Usa* 85, 9194-1006.
- McMichael, A. J., Ting, A., Zweerink, H. J. and Askonas, B. A. (1977). HLA restriction of cell-mediated lysis of influenza virus-infected human cells. *Nature* 270, 524-6.
- McMichael, A. J. and Walker, B. D. (1994). Cytotoxic T lymphocyte epitopes : implications for HIV vaccines. *AIDS* 8 (*suppl. 1*), S155-173.
- Medzhitov, R. and Janeway, C. A., Jr. (1997). Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91, 295-8.
- Mellors, J. W., Kingsley, L. A., Rinaldo, C., Todd, J., Hoo, V., Kokka, R. and Gupta, P. (1995). Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann. Intern. Med.* 122, 573-579.
- Mellors, J. W., Rinaldo, C. R., Jr., Gupta, P., White, R. M., Todd, J. A. and Kingsley, L. A. (1996). Prognosis in HIV-1 infection predicted by the quantity of virus in plasma [published erratum appears in *Science* 1997 Jan 3;275(5296):14]. *Science* 272, 1167-70.

Meyaard, L., Otto, S. A., Keet, I. P. M., Van Lier, R. A. W. and Miedema, F. (1994). Changes in cytokine secretion patterns of CD4+ T-cell clones in HIV infection. *Blood* 12, 4262-4268.

Meyerhans, A., Dadaglio, G., Vartanian, J. P., Langlade-Demoyen, P., Frank, R., Asjo, B., Plata, F. and Wain-Hobson, S. (1991). In vivo persistence of a HIV-1-encoded HLA-B27-restricted cytotoxic T lymphocyte epitope despite specific in vitro reactivity. *Eur J Immunol* 21, 2637-40.

Miller, R. A. and Stutman, O. (1984). T cell repopulation from functionally restricted splenic progenitors: 10,000-fold expansion documented by using limiting dilution analyses. *J Immunol* 133, 2925-32.

Montaner, L. and Gordon, S. (1995). Th2 down-regulation of macrophage HIV-1 replication. *Science* 267, 538-9.

Monteiro, J., Hingorani, R., Choi, I. H., Silver, J., Pergolizzi, R. and Gregersen, P. K. (1995). Oligoclonality in the human CD8+ T cell repertoire in normal subjects and monozygotic twins: implications for studies of infectious and autoimmune diseases. *J Immunol* 1, 614-624.

Moore, J. P., Cao, Y., Ho, D. D. and Koup, R. A. (1994). Development of the anti-gp120 antibody response during seroconversion to human immunodeficiency virus type 1. *J-Virol* 68, 5142-55.

Moskophidis, D., Laine, E. and Zinkernagel, R. M. (1993). Peripheral clonal deletion of antiviral memory CD8+ T cells. *Eur-J-Immunol* 23, 3306-11.

Moskophidis, D., Lechner, F., Pircher, H. and Zinkernagel, R. M. (1993). Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 362, 758-61.

- Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J-Immunol* 136, 2348-57.
- Mosmann, T. R. and Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 17, 138-46.
- Moss, P. A. H., Rowland-Jones, S. L., Frodsham, P. M., McAdam, S., Giangrande, P., McMichael, A. J. and Bell, J. I. (1995). Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors. *Proc Natl Acad Sci USA* 92, 5773-5777.
- Musey, L., Hughes, J., Schacker, T., Shea, T., Corey, L. and McElrath, M. J. (1997). Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection . *N Engl J Med* 337, 1267-74.
- Nixon, D. F., Townsend, A. R. M., Elvin, J. G., Rizza, C. R., Gallwey, J. and McMichael, A. J. (1988). HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature* 336, 484-487.
- Nowak, M. A. and Bangham, C. R. M. (1996). Population dynamics of immune responses to persistent viruses. *Science* 272, 74-79.
- O'Brien, T. R., Winkler, C., Dean, M., Nelson, J. A., Carrington, M., Michael, N. L. and White, G. C. n. (1997). HIV-1 infection in a man homozygous for CCR5 delta 32 [letter]. *Lancet* 349, 1219.
- Oberlin, E., Amara, A., Bachelier, F., Bessia, C., Virelizier, J.-L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D., Loetscher, M., Baggiolini, M. and Moser, B. (1996). The CXC chemokine SDF-1 is the ligand for

LESTR/fusin and prevents infection by T-cell-line adapted HIV-1. *Nature* 382, 833-835.

Ogg, G. S., Jin, X., Bonhoeffer, S., Dunbar, P. R., Nowak, M. A., Monard, S., Segal, J. P., Cao, Y., Rowland-Jones, S. L., Cerundolo, V., Hurley, A., Markowitz, M., Ho, D. D., Nixon, D. F. and McMichael, A. J. (1998). Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279, 2103-6.

Paillard, F., Sterkers, G. and Vaquero, C. (1990). Transcriptional and post-transcriptional regulation of TCR, CD4 and CD8 gene expression during activation of normal human T lymphocytes. *EMBO* 9, 1867-1872.

Pal, R., Garzino-Demo, A., Markham, P. D., Burns, J., Brown, M., Gallo, R. C. and DeVico, A. L. (1997). Inhibition of HIV-1 infection by the beta-chemokine MDC. *Science* 278, 695-8.

Pantaleo, G., Demarest, J. F., Soudeyns, H., Graziosi, C., Denis, F., Adelsberger, J. W., Borrow, P., Saag, M. S., Shaw, G. M., Sekaly, R. P. and Fauci, A. S. (1994). Major expansion of CD8⁺ T cells with a predominant V-beta usage during the primary immune response to HIV. *Nature* 370, 463-467.

Pantaleo, G. and Fauci, A. S. (1995). New concepts in the immunopathogenesis of HIV infection. *Annu Rev Immunol* 13, 487-512.

Papadopoulos, E. B., Ladanyi, M., Emanuel, D., Mackinnon, S., Boulad, F., Carabisi, M. H., Castro-Malaspina, H., Childs, B. H., Gillio, A. P., Small, T. N., Young, J. W., Kernan, N. A. and O'Reilly, R. J. (1994). Infusions of donor leucocytes to treat EBV-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *New Engl. J. Med.* 330, 1185-1191.

Pape, K. A., Kearney, E. R., Khoruts, A., Mondino, A., Merica, R., Chen, Z. M., Ingulli, E., White, J., Johnson, J. G. and Jenkins, M. K. (1997). Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo . *Immunol Rev* 156, 67-78.

Parker, C. E., Daenke, S., Nightingale, S. and Bangham, C. R. (1992). Activated, HTLV-1-specific cytotoxic T-lymphocytes are found in healthy seropositives as well as in patients with tropical spastic paraparesis. *Virology* 188, 628-36.

Patterson, S. and Knight, S. C. (1987). Susceptibility of human peripheral blood dendritic cells to infection by human immunodeficiency virus. *J Gen Virol* 68, 1177-81.

Pedersen, C., Dickmeiss, E., Gaub, J., Ryder, L. P., Platz, P., Lindhardt, B. O. and Lundgren, J. D. (1990). T-cell subset alterations and lymphocyte responsiveness to mitogens and antigen during severe primary infection with HIV: a case series of seven consecutive HIV seroconverters. *Aids* 4, 523-6.

Perelson, A. S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M. and Ho, D. D. (1997). Decay characteristics of HIV-1-infected compartments during combination therapy . *Nature* 387, 188-91.

Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M. and Ho, D. D. (1996). HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science* 271, 1582-6.

Phillips, R. E., Rowland-Jones, S. L., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R., Rizza, C. R. and McMichael, A. J. (1991). Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* 354, 453-9.

Piatak, M., Saag, M. and Yang, L. (1993). High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259, 1749-54.

Pircher, H., Moskhidish, A., Rohrer, U., Burki, K., Hengartner, H. and Zinkernagel, R. M. (1990). Viral escape by selection of cytotoxic T cell-resistant variants in vivo. *Nature* 346, 629-233.

Plata, F., Autran, B., Martins, L. P., Wain-Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J. M. and Debre, P. (1987). AIDS virus specific cytotoxic T lymphocytes in lung disorders. *Nature* 328, 348-351.

Podack, E. R., Hengartner, H. and Lichtenheld, M. G. (1991). A central role of perforin in cytotoxicity? *Annu Rev Immunol* 9, 129-57.

Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R. and Phillips, R. E. (1997). Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc Natl Acad Sci U S A* 94, 1890-5.

Price, D. A., Sewell, A. K., Dong, T., Tan, R., Goulder, P. J., Rowland-Jones, S. L. and Phillips, R. E. (1998). Antigen-specific release of beta-chemokines by anti-HIV-1 cytotoxic T lymphocytes. *Curr Biol* 8, 355-8.

Quinnan, G. V., Manischewitz, J. E. and Ennis, P. A. (1980). Role of cytotoxic T lymphocytes in murine cytomegalovirus infection. *J-Gen-Virol* 47, 503-8.

Rebai, N., Pantaleo, G., Demarest, J. F., Ciurli, C., Soudeyins, H., Adelsberger, J. W., Vaccarezza, M., Walker, R. E., Sekaly, R. P. and Fauci, A. S. (1994). Analysis of the T-cell receptor beta-chain variable-region (V beta) repertoire in monozygotic twins discordant for human immunodeficiency virus: evidence for perturbations of

specific V beta segments in CD4+ T cells of the virus-positive twins. *Proc Natl Acad Sci U S A* 91, 1529-33.

Reddehase, M. J., Mutter, W., Munch, K., Buhring, H. J. and Koszinowski, U. H. (1987). CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J-Virol* 61, 3102-8.

Reusser, P., Riddell, S. R., Meyers, J. D. and Greenberg, P. D. (1991). Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood* 78, 1373-80.

Riddell, S. R., Elliott, M., Lewinsohn, D. A., Gilbert, M. J., Wilson, L., Manley, S. A., Lupton, S. D., Overell, R. W., Reynolds, T. C., Corey, L. and Greenberg, P. D. (1996). T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients . *Nat-Med* 2, 216-23.

Riddell, S. R. and Greenberg, P. D. (1995). Cellular adoptive immunotherapy after bone marrow transplantation. *Cancer Treat Res* 76, 337-69.

Riddell, S. R. and Greenberg, P. D. (1995). Principles for adoptive T cell therapy of human viral diseases. *Annu Rev Immunol* 13, 545-86.

Riddell, S. R., Watanabe, K. S., Goodrich, J. M., Li, C. R., Agha, M. E. and Greenberg, P. D. (1992). Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. 257, 238-241.

Rinaldo, C. J., Beltz, L. A., Huang, X. L., Gupta, P., Fan, Z. and Torpey, D. r. (1995). Anti-HIV type 1 cytotoxic T lymphocyte effector activity and disease progression in the first 8 years of HIV type 1 infection of homosexual men. *Aids Res Hum Retroviruses* 11, 481-9.

Riviere, Y., McChesney, M. B., Porrot, F., Tanneau, S. F., Sansonetti, P., Lopez, O., Pialoux, G., Feuillie, V., Mollereau, M., Chamaret, S. and et, a. l. (1995). Gag-specific cytotoxic responses to HIV type 1 are associated with a decreased risk of progression to AIDS-related complex or AIDS. *Aids Res Hum Retroviruses* 11, 903-7.

Riviere, Y., Tanneau, S. F., Regnault, A., Lopez, O., Sansonetti, P., Guy, B., Kieny, M. P., Fournel, J. J. and Montagnier, L. (1989). Multiple cytotoxic effector cells are induced by infection with the human immunodeficiency virus. *Res Immunol* 140, 110-5.

Rocha, B. (1987). Population kinetics of precursors of IL 2-producing peripheral T lymphocytes: evidence for short life expectancy, continuous renewal, and post-thymic expansion. *J Immunol* 139, 365-372.

Rocha, B., Dautigny, N. and Pereira, P. (1989). Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. *Eur J Immunol* 19, 905-11.

Rock, K. L. and Clark, K. (1996). Analysis of the role of MHC class II presentation in the stimulation of cytotoxic T lymphocytes by antigens targeted into the exogenous antigen-MHC class I presentation pathway. *J-Immunol* 156, 3721-6.

Rock, K. L. (1996). A new foreign policy: MHC class I molecules monitor the outside world. *Immunol Today* 17, 131-7.

Romagnani, S., Del Prete, G., Manetti, R., Ravina, A., Annunziato, F., De Carli, M., Mazzetti, M., Piccinni, M.-P., D'Elia, M., Parronchi, P., Sampognaro, S. and Maggi, E. (1994). Role of Th1/Th2 cytokines in HIV infection. *Immunol. Rev.* 140, 73-92.

Rook, A. H., Smith, W. J., Burdick, J. F., Manischewitz, J. F., Frederick, W., Siegel, J. P., Williams, G. M. and Quinnan, G. V. (1984). Virus-specific cytotoxic lymphocyte responses are predictive of the outcome of cytomegalovirus infection of renal transplant recipients. *Transplant Proc* 16, 1466-9.

Rooney, C. M., Smith, C. A., Ng, C. Y. C., Loftin, S., Li, C., Krance, R. A., Brenner, M. K. and Heslop, H. E. (1995). Use of gene-modified virus-specific T lymphocytes to control EBV-related lymphoproliferation. *Lancet* 345, 9-13.

Roos, M., De Leeuw, N., Claessen, F., Huisman, H., Kootstra, N., Meyaard, L., Schellekens, P., Schuitemaker, H. and Miedema, F. (1994). Viro-immunological studies in acute HIV-1 infection. *AIDS* 8, 1533-1538.

Roques, P. A., Gras, G., Parnet-Mathieu, F., Mabondzo, A. M., Dollfus, C., Narwa, R., Marce, D., Tranchot-Diallo, J., Herve, F., Lasfargues, G. and et al. (1995). Clearance of HIV infection in 12 perinatally infected children: clinical, virological and immunological data . *Aids* 9, F19-26.

Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A. and Walker, B. D. (1997). Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia . *Science* 278, 1447-50.

Rosenberg, S. A., Lotze, M. T., Muul, L. M., Chang, A. E., Avis, F. P., Leitman, S., Linehan, W. M., Robertson, C. N., Lee, R. E., Rubin, J. T. and et al. (1987). A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 316, 889-97.

Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E., Ettinghausen, S. E., Matory, Y. L., Skibber, J. M., Shiloni, E., Vetto, J. T. and et al. (1985). Observations on the systemic administration of autologous lymphokine-

activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med* 313, 1485-92.

Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C., Seipp, C. A. and et al. (1988). Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report . *N Engl J Med* 319, 1676-80.

Rosenberg, S. A., Spiess, P. and Lafreniere, R. (1986). A new approach to the adoptive immunotherapy of cancer with tumor- infiltrating lymphocytes. *Science* 233, 1318-21.

Rosenberg, Z. F. and Fauci, A. S. (1990). Immunopathogenic mechanisms of HIV infection: cytokine induction of HIV expression. *Immunol. Today* 11, 176-180.

Rouvier, E., Luciani, M. F. and Golstein, P. (1993). Fas involvement in Ca^{2+} -independent T cell-mediated cytotoxicity. *J Exp Med* 177, 195-200.

Rowe, M., Rowe, D. T., Gregory, C. D., Young, L. S., Farrelli, P. J., Rupani, H. and Rickinson, A. B. (1987). Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma. *EMBO J.* 6, 2743.

Rowe, M., Young, L. S., Crocker, J., Stokes, H., Henderson, S. and Rickinson, A. B. (1991). Epstein-Barr virus (EBV)-associated lymphoproliferative disease in the SCID mouse model: implications for the pathogenesis of EBV-positive lymphomas in man. *J. Exp. Med.* 173, 147-158.

Rowland-Jones, S. and McMichael, A. (1993). Cytotoxic T lymphocytes in HIV infection. *Semin. Virol.* 4, 83-94.

Rowland-Jones, S., Tan, R. and McMichael, A. (1997). Role of cellular immunity in protection against HIV infection. *Adv Immunol* 65, 277-346.

Rowland-Jones, S. L., Nixon, D. F., Aldhous, M. C., Gotch, F., Ariyoshi, K., Hallam, N., Kroll, J. S., Froebel, K. and McMichael, A. J. (1993). HIV-specific CTL activity in an HIV-exposed but uninfected infant. *341*, 860-861.

Rowland-Jones, S. L., Sutton, J., Ariyoshi, K., Dong, T., Gotch, F. M., McAdam, S., Whitby, D., Sabally, S., Gallimore, A., Corrah, T., Takiguchi, M., Schultz, T., McMichael, A. J. and Whittle, H. (1995). HIV-specific cytotoxic T cells in HIV-exposed but uninfected Gambian women. *Nature Med* 1, 59-64.

Safrit, J. T., Andrews, C. A., Zhu, T., Ho, D. D. and Koup, R. A. (1994). Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: recognition of autologous virus sequences within a conserved immunodominant epitope. *J Exp Med* 179, 463-72.

Safrit, J. T. and Koup, R. A. (1995). The immunology of primary HIV infection : which immune responses control HIV replication ? *Curr. Opin. Immunol.* 7, 456-461.

Sarin, A., Williams, M. S., Alexander-Miller, M. A., Berzofsky, J. A., Zacharchuk, C. M. and Henkart, P. A. (1997). Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. *Immunity* 6, 209-15.

Sauzet, J. P., Deprez, B., Martinon, F., Guillet, J. G., Gras Masse, H. and Gomard, E. (1995). Long-lasting anti-viral cytotoxic T lymphocytes induced in vivo with chimeric-multirestricted lipopeptides. *Vaccine* 13, 1339-45.

- Schwartz, R. S. (1998). Direct visualization of antigen-specific cytotoxic T cells--a new insight into immune defenses. *N Engl J Med* 339, 1076-8.
- Schwartz, S., Felber, B. K., Benko, D. M., Fenyo, E. M. and Pavlakis, G. N. (1990). Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J Virol* 64, 2519-29.
- Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B. and Mak, T. W. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261, 609-12.
- Shaper, E. G. and Mullins, J. I. (1993). Rates of amino acid change in the envelope protein correlate with pathogenicity of primate lentiviruses. *J-Mol-Evol* 37, 57-65.
- Shirai, M., Chen, M., Arichi, T., Masaki, T., Nishioka, M., Newman, M., Nakazawa, T., Feinstone, S. M. and Berzofsky, J. A. (1996). Use of intrinsic and extrinsic helper epitopes for in vivo induction of anti-hepatitis C virus cytotoxic T lymphocytes (CTL) with CTL epitope peptide vaccines. *J-Infect-Dis* 173, 24-31.
- Shirai, M., Pendleton, C. D., Ahlers, J., Takeshita, T., Newman, M. and Berzofsky, J. A. (1994). Helper-cytotoxic T lymphocyte (CTL) determinant linkage required for priming of anti-HIV CD8+ CTL in vivo with peptide vaccine constructs. *J-Immunol* 152, 549-56.
- Sondel, P. M., Hank, J. A., Kohler, P. C., Chen, B. P., Minkoff, D. Z. and Molenda, J. A. (1986). Destruction of autologous human lymphocytes by interleukin 2-activated cytotoxic cells. *J Immunol* 137, 502-11.
- Stein, B., Kramer, M., Rahmsdorf, H. J., Ponta, H. and Herrlich, P. (1989). UV-induced transcription from the human immunodeficiency virus type 1 (HIV-1) long

terminal repeat and UV-induced secretion of an extracellular factor that induces HIV-1 transcription in nonirradiated cells. *J Virol* 63, 4540-4.

Street, N. E., Schumacher, J. H., Fong, T. A. T., Bass, H., Fiorentino, D. F., Leverah, J. A. and Mosmann, T. R. (1990). Heterogeneity of mouse helper T cells: evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. *J Immunol* 144, 1629.

Tan, R., Teh, S. J., Ledbetter, J. A., Linsley, P. S. and Teh, H. S. (1992). B7 costimulates proliferation of CD4-8+ T lymphocytes but is not required for the deletion of immature CD4+8+ thymocytes. *J Immunol* 149, 3217-24.

Theodorou, I., Meyer, L., Magierowska, M., Katlama, C., Rouzioux, C. and Study Group, t. S. (1997). HIV-1 infection in an individual homozygous for CCR5d32. *Lancet* 349, 1219-20.

Topalian, S. L., Muul, L. M., Solomon, D. and Rosenberg, S. A. (1987). Expansion of human tumor infiltrating lymphocytes for use in immunotherapy trials. *J-Immunol-Methods* 102, 127-41.

Topalian, S. L., Solomon, D., Avis, F. P., Chang, A. E., Freerksen, D. L., Linehan, W. M., Lotze, M. T., Robertson, C. N., Seipp, C. A., Simon, P. and et al. (1988). Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2: a pilot study. *J Clin Oncol* 6, 839-53.

Toso, J. F., Chen, C.-H., Mohr, J. R., Piglia, L., Oei, C., Ferrari, G., Greenberg, M. L. and Weinhold, K. J. (1995). Oligoclonal CD8 lymphocytes from persons with asymptomatic HIV-1 infection inhibit HIV-1 replication. *JID* 172, 964-973.

Townsend, A. and Bodmer, H. (1989). Antigen recognition by class I -restricted cytotoxic T lymphocytes. *Ann Rev Immunol* 7, 601-624.

Townsend, A., Rothbard, J., Gotch, F., Bahadur, B., Wraith, D. and McMichael, A. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44, 959-968.

Tripp, R. A., Hou, S., McMickle, A., Houston, J. and Doherty, P. C. (1995). Recruitment and proliferation of CD8+ T cells in respiratory virus infections. *J-Immunol* 154, 6013-21.

Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A. R. M. and Kelly, A. (1990). Sequences encoded in the class II region of the MHC related to the ABC superfamily of transporters. *Nature* 348, 741-744.

Tsomides, T. J., Aldovini, A., Johnson, R. P., Walker, B. D., Young, R. A. and Eisen, H. N. (1994). Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. *J Exp Med* 180, 1283-93.

Tsubota, H. C., Lord, C. I., Watkins, D. I., Morimoto, C. and Letvin, N. L. (1989). A cytotoxic T lymphocyte inhibits acquired immunodeficiency syndrome virus replication in peripheral blood lymphocytes. *J. Exp. Med.* 169, 1421-1434.

Van Bleek, G. M. and Nathenson, S. M. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H2Kb molecule. *Nature* 348, 213.

Vigano, A., Principi, N., Crupi, L., Onorato, J., Vincenzo, Z. G. and Salvaggio, A. (1995). Elevation of IgE in HIV-infected children and its correlation with the progression of disease. *J-Allergy-Clin-Immunol* 95, 627-32.

Vilmer, E., Rhodes Feuillette, A., Rabian, C., Benbunan, M., Meletis, J., Devergie, A., Bourrhis, J. H., Gluckman, J. C., Chermann, J. C. and Gluckman, E. (1987). Clinical and immunological restoration in patients with AIDS after marrow transplantation, using lymphocyte transfusions from the marrow donor. *Transplantation* 44, 25-9.

von Herrath, M. G., Yokoyama, M., Dockter, J., Oldstone, M. B. and Whitton, J. L. (1996). CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. *J-Virol* 70, 1072-9.

Vyakarnam, A., Matear, P. M., Martin, S. J. and Wagstaff, M. (1995). Th1 cells specific for HIV-1 gag p24 are less efficient than Th0 cells in supporting HIV replication, and inhibit virus replication in Th0 cells. *Immunology* 86, 85-96.

Wagner, L., Yang, O. O., Garcia-Zepeda, E. A., Ge, Y., Kalams, S. A., Walker, B. D., Pasternack, M. S. and Luster, A. D. (1998). Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans. *Nature* 391, 908-11.

Wagtmann, N., Biassoni, R., Cantoni, C., Verdiani, S., Malnati, M. S., Vitale, M., Bottino, C., Moretta, L., Moretta, A. and Long, E. O. (1995). Molecular clones of the p58 NK cell receptor reveal immunoglobulin- related molecules with diversity in both the extra- and intracellular domains. *Immunity* 2, 439-49.

Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S. and Schooley, R. T. (1987). HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* 328, 345-8.

Walker, C. M., Moody, D. J., Stites, D. P. and Levy, J. A. (1986). CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 234, 1563-1566.

Walker, R., Blaese, R. M., Carter, C. S., Chang, L., Klein, H., Lane, H. C., Leitman, S. F., Mullen, C. A. and Larson, M. (1993). A study of the safety and survival of the adoptive transfer of genetically marked syngeneic lymphocytes in HIV-infected identical twins. *Hum Gene Ther* 4, 659-80.

Walker, S., Hagemeyer, C., Sissons, J. G. and Sinclair, J. H. (1992). A 10-base-pair element of the human immunodeficiency virus type 1 long terminal repeat (LTR) is an absolute requirement for transactivation by the human cytomegalovirus 72-kilodalton IE1 protein but can be compensated for by other LTR regions in transactivation by the 80-kilodalton IE2 protein. *J Virol* 66, 1543-50.

Wallace, L. E., Rickinson, A. B., Rowe, M. and Epstein, M. A. (1982). Epstein-Barr virus-specific cytotoxic T-cell clones restricted through a single HLA antigen. *Nature* 297, 413-5 issn: 0028-0836.

Wallace, L. E., Rowe, M., Gaston, J. S. H., Rickinson, A. B. and Epstein, M. A. (1982). Cytotoxic T cell recognition of Epstein-Barr virus-infected B cells. III. Establishment of HLA-restricted cytotoxic T cell lines using interleukin 2. *Eur. J. Immunol.* 12, 1012-1017.

Walsh, C. M., Matloubian, M., Liu, C. C., Ueda, R., Kurahara, C. G., Christensen, J. L., Huang, M. T., Young, J. D., Ahmed, R. and Clark, W. R. (1994). Immune function in mice lacking the perforin gene. *Proc Natl Acad Sci U S A* 91, 10854-8.

Walter, E. A., Greenberg, P. D., Gilbert, M. J., Finch, R. J., Watanabe, K. S., Thomas, E. D. and Riddell, S. R. (1995). Reconstitution of cellular immunity against

cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* 333, 1038-44.

Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H. and et al. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373, 117-22.

Weiss, R. A. (1996). HIV receptors and the pathogenesis of AIDS. *Science* 272, 1885-6.

Wiertz, E. J., Tortorella, D., Bogoy, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A. and Ploegh, H. L. (1996). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction . *Nature* 384, 432-8.

Wiertz, E. J. H. H., Jones, T. R., Sun, L., Bogoy, M., Geuze, H. J. and Ploegh, H. L. (1996). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* 84, 769-779.

Wolfs, T. F., de Jong, J. J., Van den Berg, H., Tijnagel, J. M., Krone, W. J. and Goudsmit, J. (1990). Evolution of sequences encoding the principal neutralization epitope of human immunodeficiency virus 1 is host dependent, rapid, and continuous. *Proc-Natl-Acad-Sci-U-S-A* 87, 9938-42.

Wolinsky, S. M., Korber, B. T. M., Neumann, A. U., Daniels, M., Kunstman, K. J., Whetsell, A. J., Furtado, M. R., Cao, Y., Ho, D. D., Safrit, J. T. and Koup, R. A. (1996). Adaptive evolution of Human Immunodeficiency Virus-Type 1 during the natural course of Infection. *Science* 272, 537-540.

Wolthers, K. C., Bea, G., Wisman, A., Otto, S. A., de Roda Husman, A. M., Schaft, N., de Wolf, F., Goudsmit, J., Coutinho, R. A., van der Zee, A. G., Meyaard, L. and

Miedema, F. (1996). T cell telomere length in HIV-1 infection: no evidence for increased CD4+ T cell turnover. *Science* 274, 1543-7.

Xu, X.-N., Screaton, G. R., Gotch, F. M., Dong, T., Tan, R., Almond, N., Walker, B., Stebbings, R., Kent, K., Nagata, S., Stott, J. E. and McMichael, A. J. (1997). Evasion of cytotoxic T lymphocyte (CTL) responses by nef-dependent induction of fas ligand (CD95L) expression on simian immunodeficiency virus-infected cells. *J Exp Med* 186, 7-16.

Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S. P., Aleksander, I. and Mak, T. W. (1984). A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature* 308, 145-9.

Yang, O., Kalams, S., Rosenweig, M., Trocha, A., Jones, N., Koziel, M., Walker, B. D. and Johnson, R. P. (1996). Efficient lysis of HIV-1-infected cells by cytotoxic T lymphocytes. *J. Virol.* 70, 5799-5806.

Yang, O. O., Kalams, S. A., Trocha, A., Cao, H., Luster, A., Johnson, R. P. and Walker, B. D. (1997). Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J Virol* 71, 3120-8.

Yang, O. O., Tran, A. C., Kalams, S. A., Johnson, R. P., Roberts, M. R. and Walker, B. D. (1997). Lysis of HIV-1-infected cells and inhibition of viral replication by universal receptor T cells. *Proc Natl Acad Sci U S A* 94, 11478-83.

Yap, K. L., Ada, G. L. and McKenzie, I. F. C. (1978). Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature* 273, 238-239.

York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L. and Johnson, D. C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* 77, 525-35.

Young, J. W. and Steinman, R. M. (1990). Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells. *J-Exp-Med* 171, 1315-32.

Young, L., Alfieri, C., Hennessey, K., Evans, H., O'Hara, C., Anderson, K. C., Ritz, J., Shapiro, R. S., Rickinson, A., Kieff, E. and Cohen, J. I. (1989). Expression of Epstein-Barr virus transformation associated genes in tissues of patients with EBV lymphoproliferative disease. *New. Eng. J. Med.* 321, 1080.

Zack, J. A., Arrigo, S. J., Weitsman, S. R., Go, A. S., Haislip, A. and Chen, I. S. (1990). HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* 61, 213-22.

Zack, J. A., Cann, A. J., Lugo, J. P. and Chen, I. S. (1988). HIV-1 production from infected peripheral blood T cells after HTLV-I induced mitogenic stimulation. *Science* 240, 1026-9.

Zhang, L. Q., MacKenzie, P., Cleland, A., Holmes, E. C., Leigh Brown, A. J. and Simmonds, P. (1993). Selection for specific sequences in the external envelope protein of HIV-1 upon primary infection. *J. Virol.* 67, 3345-3356.

Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A. and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. *Science* 261, 1179-1181.

Zinkernagel, R. M. and Doherty, P. C. (1974). Restriction of an in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within asyngeneic or semiallogeneic system. 248, 701-702.

Zinkernagel, R. M. and Doherty, P. C. (1975). H-2 compatibility requirement for T-cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. J.Exp.Med. 141, 1427-1436.

Zweerink, H. J., Coutneidge, S. A., Skehel, J. J., Crumpton, M. J. and Askonas, B. A. (1977). Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. 267, 354-356.